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EFFECT OF VARIOUS DRUGS ON EXPERIMENTAL ASTHMA PRODUCED IN GUINEA PIGS BY EXPOSURE TO ATOMIZED HISTAMINE

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We have recently published data (1) indicating that benzhydryl alkamine ethers and other anti-histamine agents are markedly effective in reducing the severity of bronchoconstriction following exposure of guinea pigs to atomized histamine solutions. In view of the fact that this technique has been used only to a limited extent it is important to determine whether the drugs found to be effective are limited to those employed clinically by virtue of their recognized efficacy in bronchial asthma and to those which possess a demonstrable anti-histamine or antispasmodic action. A comparison was made of the effectiveness of bronchodilator drugs, uterine and gastrointestinal antispasmodics, and other types of drugs in experimental asthma induced with atomized histamine.

METHODS. Prophylactic treatment was effected by intraperitoneal administration of drugs 15 minutes before subjecting guinea pigs to atomized histamine under conditions as previously described (1). To control possible daily variation a separate group of control animals was utilized in each daily experiment. The incidence of asphyxial deaths due to severe bronchoconstriction in the untreated control animals consistently amounted to 75 to 100 per cent, whereas a reduction of mortality to 25 or 65 per cent in groups of 12 to 20 treated animals constituted a significant difference which suggested that the severity of bronchoconstriction had been diminished. Untreated, control animals usually died in four to ten minutes. Since administration of histamine was by inhalation, it is conceivable that drugs which increase or decrease the respiratory exchange, and therefore the amount of histamine inhaled, would increase or lessen mortality. It is important, therefore, to note the effects of drugs known to have respiratory stimulant or depressant properties.

RESULTS. As stated in our previous publication (1), an activity index of unity was given to aminophylline (table 1) which, with a dose of 50 mgm./kgm. administered intraperitoneally, significantly reduced the mortality to 50 or 30 per cent. The minimal effective dose of all drugs was considered as the least dose which yielded a reduction in percentage mortality to 50 or 30 per cent in groups of 12 to 20 guinea pigs. The activity index of any drug is the ratio of minimal effective dose of aminophylline (50 mgm./kgm.) to the minimal effective dose of the given drug. Thus, papaverine was effective at a dosage level of 25 mgm./kgm. and the activity index therefore became 50/25 or 2.0. No activity index was ascribed to a drug if doses of reasonable magnitude failed to exert beneficial effects.

A. *Antispasmodics.* Data presented in table 1 indicate that papaverine and

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TABLE 1

Effect of drugs on mortality rate of guinea pigs exposed to atomized histamine

CHEMICAL COMPOUND	TREATED*			UNTREATED CONTROLS		DECREASE IN PERCENTAGE MORTALITY	P†	ACTIVITY INDEX
	Dose	Mortality		Mortality				
		Ratio	Per cent	Ratio	Per cent			
	mgm./kgm.							
Theophylline-ethylenediamine (Aminophylline)	50	10/20	50	16/20	80	30	0.05	1
Papaverine Hydrochloride	25	10/16	63	16/16	100	37	<0.01	2
Atropine Sulfate	15	16/28	57	28/31	90	33	<0.01	3
1-Methyl-4-phenylpiperidine-4-carboxylic acid ethyl ester hydrochloride (Demerol; isonipecaine)	25	4/12	33	12/12	100	67	<0.01	
	12.5	3/12	25	10/12	83	58	0.01	
	6	10/20	50	18/20	90	40	<0.01	8
	3	16/20	80	18/20	90	10		
β-Dimethylaminoethyl benzhydryl ether hydrochloride (Benadryl)‡	3	9/20	45	19/20	95	50		
	1.5	7/20	35	18/20	90	55	<0.01	33
Epinephrine (Adrenalin Chloride)	0.3	5/12	42	14/15	93	51	<0.01	
	0.1	8/12	66	12/12	100	34	0.02	500
	0.05	17/20	85	18/20	90	5		
Ephedrine Hydrochloride	20	19/20	95	17/20	85			
	10	19/20	95	17/20	85			
2-Aminoheptane Sulfate (Tuamine Sulfate)	5	14/20	70	15/20	75	5		
Caffeine Sodium Benzoate	50	17/20	85	16/20	80			
	25	19/20	95	19/20	95			
β-Diethylaminoethyl diphenylacetate hydrochloride (Trasentin)	50	11/16	69	13/16	81	12	>0.5	
β-Diethylaminoethylfluorene-9-carboxylate hydrochloride (Pavatrine)	50	19/20	95	18/20	90			
	25	15/16	94	17/20	85			
γ-Diethylamino-β,β-dimethylpropyl-dl-tropate phosphate (Syntropan)	30	10/12	83	13/16	81			

* All compounds administered by intraperitoneal injection.

† P values from Fisher's Table. Value less than 0.05 indicative of significant difference.

‡ Data from reference No. 1.

§ Animals exhibited ataxia and slight degree of depression.

¶ Definite depression; most of animals prostrate.

TABLE 1—*Continued*

CHEMICAL COMPOUND	TREATED*			UNTREATED CONTROLS		DECREASE IN PERCENTAGE MORTALITY	P†	ACTIVITY INDEX
	Dose	Mortality		Mortality				
		Ratio	Per cent	Ratio	Per cent			
	mgm./kgm.							
Physostigmine Salicylate	0.75	16/16	100	23/24	96			
Ergotamine Tartrate	*0.15* 1.5	*12/12* 12/12	*100* 100	*12/12* 12/12	*100* 100			
Morphine Sulfate	25	18/20	90	20/20	100	10		
Pentobarbital Sodium	10§ 20¶	19/20 12/20	95 60	19/20 17/20	95 85	 25	 0.07	
2-Butoxy-4-(β-diethylaminoethylamido) carboxy quinoline hydrochloride (Nupercaine)	5	20/20	100	19/20	95			
Diethylaminoethyl p-aminobenzoate hydrochloride (Procaine)	25	19/20	95	19/20	95			

atropine with activity indices of 2 and 3, respectively, are both more effective than aminophylline in reducing the mortality rate of guinea pigs exposed to atomized histamine. Demerol (isonipecaine) was very efficacious as indicated by the reduction in mortality following a dose of only 6.0 mgm./kgm., thus giving an activity index of 8. Previously published data (1) indicating the marked effectiveness of Benadryl[2] have been included in table 1. With the doses employed, Trasentin, Pavatrine, and Syntropan were ineffective in relieving histamine-induced bronchoconstriction.

B. *Pressor amines and respiratory depressants.* Epinephrine reduced mortality but administration of a comparatively large dose (0.3 mgm./kgm.) of such a potent drug did not completely eliminate mortality. In contrast, tolerated doses of a compound such as Benadryl which antagonizes histamine in a more specific manner (11) reduced mortality to zero and suppressed symptoms to an appreciable degree (1). Although epinephrine is probably the most potent drug (on an absolute dosage basis) which decreases the severity of histamine-induced bronchoconstriction, it lacks the specificity of action possessed by a number of antihistamine compounds.

Ephedrine was ineffective in experimental asthma even though a wide range of doses were administered both orally and intraperitoneally. Typical data are presented.

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[2] β-Dimethylaminoethyl benzhydryl ether hydrochloride.

Tuamine sulfate, a pressor amine of the aliphatic type (3), failed to alleviate histamine-induced asthma.

Drugs known to depress respiration, pentobarbital and morphine, did not reduce the mortality rate of guinea pigs which received histamine by the inhalant method.

C. *Miscellaneous drugs.* Physostigmine administration aggravated histamine shock as indicated by 100 per cent mortality and the rapidity with which the animals succumbed. In a separate experiment it was demonstrated that after treatment with physostigmine a mortality of 80 per cent of twenty animals resulted following a smaller dose of histamine which was fatal to only 50 per cent of twenty untreated guinea pigs. The detrimental effects of physostigmine in histamine shock may be referable to the potentiation of the systemic effects of acetylcholine which would be liberated in increased quantities during asphyxial convulsions. However, consideration should also be given to other factors, such as toxicity, pulmonary edema, and pharmacological properties of physostigmine which are unrelated to anti-cholinesterase activity.

It was of interest to determine the effects of a sympathicolytic agent such as ergotamine tartrate on histamine-induced asthma in view of the fact that 929F 4) and the dioxane derivatives, 883F and 933F, possess sympathicolytic and anti-histamine properties (5–7). The doses of ergotamine employed, 0.15 and 1.5 mgm./kgm., were chosen to represent sympathicomimetic and sympathicolytic doses, respectively (8). Neither dose conferred any protection.

Two local anesthetics, Procaine and Nupercaine, were found to be devoid of anti-histamine activity.

Discussion. Several antispasmodics were included in the present study in order to determine their relative effectiveness, if any, in alleviating the experimental asthma induced with atomized histamine. The neurotropic and musculotropic antispasmodics, atropine and papaverine, respectively, proved effective and are known to be capable of relieving the bronchoconstriction encountered in anaphylactic and histamine shock (2, 4, 9, 13, 14). The necessity of giving large doses of atropine probably accounts for the conflicting opinions expressed in the earlier literature (2) regarding the effectiveness of atropine in anaphylactic shock.

Trasentin, Pavatrine, and Syntropan were ineffective despite the fact that each is appreciably effective in antagonizing the spasmogenic effect of histamine on intestinal or uterine smooth muscle *in vitro* (9–11). We have found no evidence in the literature which indicates that these compounds have a prominent bronchodilator action. Nevertheless, an anti-histamine action can readily be demonstrated on smooth muscle of the intestine and uterus. In contrast, aminophylline has a comparatively weak anti-histamine action when tested on intestinal smooth muscle (12) but is effective in reducing mortality in experimental asthma and gives relief in clinical cases of bronchial asthma. These facts suggest that aminophylline has an anti-histamine action which is prominent on bronchiolar muscle. Lehman and Young (13) have recently pointed out that such salutary effects of aminophylline in histamine-induced asthma are probably not related solely to relief of bronchoconstriction but to alleviation of spasm of pulmonary blood vessels as well. Although atropine and papaverine reduced the mortality rate of guinea pigs exposed to atomized histamine, no significant selective action on bronchiolar smooth muscle can be inferred since these drugs have pronounced antispasmodic action on other tissues.

Experimental and clinical literature relating to Demerol indicate that this analgetic compound possesses sedative and antispasmodic properties (for references see 15–17). Demerol must possess a distinctive anti-histamine or bronchiolar antispasmodic action for our data reveal a marked ability to alleviate histamine-induced asthma; in fact the potency of Demerol is equalled or exceeded only by epinephrine, anti-histamine drugs such as several benzhydryl alkamine ethers (1) and the Fourneau histamine antagonists, 1571F and 929F (1, 4). These data corroborate Schaumann's demonstration (17) that Demerol alleviates histamine-induced asthma in guinea pigs. It is pertinent to note that hypodermic injection of Demerol is an effective means of treating acute attacks of bronchial asthma (15).

We were unable to demonstrate that ephedrine decreased the severity of asthma induced by inhalation of histamine. Issekutz and Genersich (18) found that ephedrine failed to alleviate even less severe asthma induced in like manner. The ineffectiveness of ephedrine may be due to the fact that respiratory stimulation increases the amount of histamine inhaled and thereby completely masks the bronchodilator properties of ephedrine. In support of this assumption is the fact that when guinea pigs were subjected to smaller quantities of atomized histamine which killed only 50 per cent of 20 untreated animals, the incidence of mortality was increased to 80 per cent in 20 animals pretreated with ephedrine (20 mgm./kgm.). On the other hand, it is not surprising that ephedrine failed to alleviate the bronchoconstriction induced with histamine since it is rated as a poor bronchodilator by those investigators (20, and refs. cited) who have studied it in isolated guinea pig lungs and anesthetized dogs.

Caffeine did not increase or decrease the severity of bronchoconstriction. Respiratory stimulation with caffeine would probably exceed that following ephedrine but the bronchodilator effect of the xanthine molecule might mask any increased lethal effect due to increased inhalation of histamine. With caffeine sodium benzoate the xanthine content and amount injected was appreciably lower than the amount in the dose of aminophylline which was considered to be the minimal effective dose.

It was deemed important to determine whether respiratory depressants would be effective in reducing the severity of asthma induced by inhalation of histamine. Pentobarbital as well as morphine proved ineffective. We have secured no evidence which indicates that respiratory depressants significantly reduce mortality. Among several hundred synthetic compounds tested, a small number relieved asthma and in each instance the compound was found capable of relaxing isolated intestinal muscle, thus demonstrating antispasmodic action. We believe, therefore, that the experimental technique employed selects only those drugs with antispasmodic or anti-histamine action and that the technique is valuable in evaluating such drugs by aiding in determining the selective action of any antispasmodic on smooth muscle in various locations.

Because of chemical similarities between antispasmodics and local anesthetics and because antispasmodics (9, 10, 19), as well as anti-histamine drugs such as the benzhydryl alkamine ethers (11), have appreciable local anesthetic action, Procaine and Nupercaine were tested for anti-histamine activity. The failure of these local anesthetics to decrease histamine shock is of experimental interest for Procaine is frequently used as a local anesthetic when intravenous injections are made in experiments dealing with histamine and anaphylactic shock. The local anesthetic, Stovaine, is reported to possess anti-histamine properties (2).

SUMMARY

Trasentin, Pavatrine, and Syntropan failed to alleviate experimental asthma induced in guinea pigs by exposing them to atomized histamine. Effective antispasmodic drugs in order of decreasing potency were: epinephrine, Benadryl, Demerol, atropine, papaverine, and aminophylline. The effect of drugs in experimental asthma should assist in selecting those with selective antispasmodic action on various types of smooth muscle.

Under the experimental conditions employed, bronchoconstriction referable to inhalation of histamine was not diminished by ergotamine tartrate, the local anesthetics, Procaine and Nupercaine, or by respiratory depressants such as morphine and pentobarbital.

Physostigmine and ephedrine increased the severity of the experimental asthma.

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THE ANTI-HISTAMINE ACTION OF ALKYLOXYTRIAZINES AND RELATED COMPOUNDS

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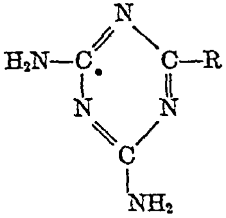
Research during the past decade has revealed that a number of synthetic organic compounds such as phenolic ethers (1, 2) and benzhydryl alkamine ethers (3–6) are effective histamine antagonists. The present report presents evidence that other ethers, alkyloxydiaminotriazines, are capable of antagonizing the action of histamine on smooth muscle of bronchioles and intestines. Twenty-four 2-substituted diaminotriazines have been tested for anti-histamine activity. These synthetic compounds[2] have the general formula,



where R represents an alkyloxy, cycloalkyloxy, aryloxy, amino, or substituted amino group.

Methods. The triazines were usually administered intraperitoneally to guinea pigs fifteen minutes before subjecting them to atomized histamine under conditions previously described (3). A few compounds of low solubility were suspended in 2 per cent gum acacia in water and administered orally in doses of 100 mgm./kgm. Compounds were considered as effective anti-histamine agents if the mortality due to severe bronchoconstriction was significantly less than the 80 to 100 per cent which obtained in groups of untreated control animals (table 1) exposed to atomized histamine during each day of experimentation. The potency of any compound was determined by finding the least dose which would reduce the percentage mortality of histamine-treated guinea pigs to 30 or 50 per cent. An activity index of unity was designated for aminophylline in which case the minimal effective dose was 50 mgm./kgm. The activity index for each drug represents the ratio of the dose of aminophylline (50 mgm./kgm.) to the minimal effective dose of the drug tested. No activity index was designated for those drugs which were ineffective with a dose of 50 mgm./kgm. intraperitoneally, or 100 mgm./kgm. orally, or with a dose known to be near the maximum amount tolerated.

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[2] We are greatly indebted to Dr. K. Banks and Mr. J. Controulis of the Chemical Research Laboratories, Parke, Davis and Company, who synthesized the compounds which formed the basis of this study.

The acute toxicity following intraperitoneal injection of 2 to 5 per cent aqueous solutions was determined for most of the compounds by administering each of five selected doses to a group of 12 mice, weighing 18 to 20 Grams. Deaths occurring during the next five days were recorded. The LD-50 was calculated from the deduced mortality obtained as indicated by Dragstedt and Lang (7, also cf. Behrens, 8).

Experiments were also made to determine the antispasmodic action of alkyloxytriazines on intestinal muscle. Several compounds found to be the most effective in experimental asthma were tested for their ability to prevent the spasmogenic effects of histamine, barium and acetylcholine. Segments of guinea pig ileum were suspended in 100 cc. of oxygenated Tyrode's solution maintained at 38°C. The spasmogenic agents employed and the final dilutions in the muscle bath were: histamine diphosphate, 1:12,500,000; barium chloride, 1:10,000; and acetylcholine bromide, 1:50,000,000. After obtaining several control contractions of equal magnitude (fig. 1) with a given spasmogenic agent, a drug was added to the bath one minute before again adding the spasmogenic agent. Repeated tests were made to determine the dilution of each compound which would inhibit the spasmogenic agents 75 to 100 per cent, i.e., almost completely prevent occurrence of spasm. Aminophylline was alternated with the other compounds in each experiment in order to directly compare relative potencies.

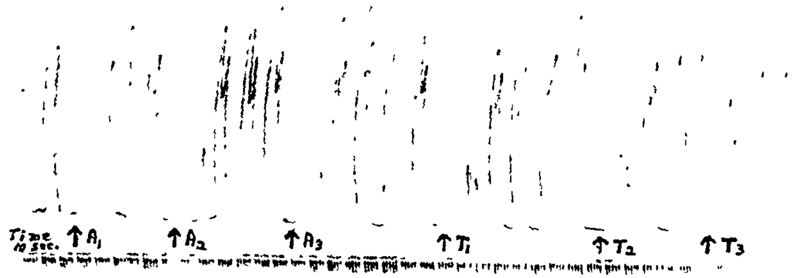

FIG. 1. SPASMOGENIC ACTION OF HISTAMINE ON INTESTINAL MUSCLE INHIBITED BY AMINOPHYLLINE AND ALKYLOXYTRIAZINES

Each contraction elicited with histamine diphosphate; continuous recording.

Muscle was washed when each contraction attained maximum height; normal response of muscle obtained immediately when test drugs were washed out.

A_1—Aminophylline, 1:5,000; no significant reduction of response to histamine.

A_2—Aminophylline, 1:2,000; response to histamine reduced 55 per cent.

A_3—Aminophylline, 1:1,666; response to histamine reduced 75 per cent.

Note increased inhibition with increased dose of aminophylline.

T_1—2-sec.-n-Butoxy-4,6-diamino-s-triazine, 1:20,000; response to histamine reduced 65 per cent.

T_2—2-Iso-propoxy-4,6-diamino-s-triazine, 1:20,000; response to histamine reduced 65 per cent.

T_3—2-n-Propoxy-4,6-diamino-s-triazine, 1:20,000; response to histamine reduced 35 per cent.

RESULTS. A. *Prevention of bronchoconstriction.* Data presented in table 1 reveal that all of the effective compounds contain an ether linkage. The most active compounds were alkyloxytriazines (nos. 3, 4, 6, 7, and 15), each being four times as potent as aminophylline in reducing mortality of histamine-treated guinea pigs, presumably by preventing bronchoconstriction. In general, activity

TABLE 1

Reduction of mortality in asthmatic guinea pigs by 2-substituted diaminotriazines

CHEMICAL COMPOUND	TREATED*				UNTREATED CONTROLS		DECREASE IN PERCENTAGE MORTALITY	P†	ACTIVITY INDEX	LD-50, MICE (I.P.)
	Dose	Route	Mortality		Mortality					
			Ratio	Per cent	Ratio	Per cent				
	mgm./kgm.									mgm./kgm.
Reference drug: Theophylline-ethylenediamine (Aminophylline)	50	I.P.	12/20	60	19/20	95	35	<0.01	1.0	260
	50	Oral	14/29	48	25/25	100	52	<0.01	1.0	
2-R-4,6-diamino-s-triazines										
1. *R*-Methoxy	50	I.P.	12/20	60	14/16	88	28	0.04	1.0	495
2. Ethoxy	25	I.P.	12/20	60	18/20	90	30	0.02	2.0	495
	50	Oral	8/16	50	13/16	81	31	0.04		
3. Propoxy	25	I.P.	6/20	30	18/20	90	60			
	12.5	I.P.	12/20	60	19/20	95	35	<0.01	4.0	520
	25	Oral	12/16	75	15/16	94	19	0.1		
4. Isopropoxy	12.5	I.P.	14/20	70	16/16	100	30	<0.01	4.0	357
	25	Oral	9/16	56	15/16	94	38	0.01		
5. Butoxy	25	I.P.	8/20	40	18/20	90	50		2.0	215
	12.5	I.P.	17/20	85	19/20	95	10	0.3		
6. Isobutoxy	25	I.P.	10/20	50	18/20	90	40			
	12.5	I.P.	10/20	50	16/20	80	30	0.05	4.0	335
7. Sec.-n-butoxy	25	I.P.	7/20	35	18/20	90	55			
	12.5	I.P.	10/20	50	16/20	80	30	0.05	4.0	410
8. β-Ethoxy-ethoxy	50	I.P.	5/12	42	14/16	88	46	0.01	1.0	
	25	I.P.	16/20	80	19/20	95	15	0.15		
9. Pentoxy	25	I.P.	12/20	60	17/20	85	25	0.07		133
10. Hexoxy	25	I.P.	8/20	40	16/18	88	48		>2.0	115
	12.5	I.P.	15/20	75	19/20	95	20	0.07		
11. Heptoxy	25	I.P.	17/20	85	18/20	90	5	0.64		55
12. Octoxy	25	I.P.	18/20	90	17/20	85				43
13 Nonoxy	25	I.P.	19/20	95	17/20	85				116

* Drugs injected intraperitoneally or given orally 15 and 30 minutes, respectively, before exposure to atomized histamine.

† P value (from Fisher's Table) less than 0.05 indicates significant difference.

TABLE 1—*Continued*

CHEMICAL COMPOUND	TREATED*				UNTREATED CONTROLS		DECREASE IN PERCENTAGE MORTALITY	P†	ACTIVITY INDEX	LD-50, MICE (I.P.)
	Dose	Route	Mortality		Mortality					
			Ratio	Per cent	Ratio	Per cent				
	mgm./kgm.									*mgm./kgm.*
14. β-Dimethylaminoethoxy	25	I.P.	14/20	70	18/20	90	20	0.1		
15. Cyclohexoxy	25	I.P.	4/20	20	18/20	90	70			
	12.5	I.P.	7/20	35	16/20	80	45	<0.01	4.0	237
	6.0	I.P.	14/20	70	16/20	80	10	0.47		
16. β-Morpholinoethoxy	100	I.P.	11/16	69	18/20	90	21	0.1		>1000
17. Phenoxy	50	I.P.	10/16	63	15/16	94	31	0.03	1.0	
18. Benzyloxy	25	I.P.	19/20	95	16/16	100	5			
19. Amino	100	Oral	11/12	92	14/16	88				
20. Anilino	100	I.P.	13/16	81	15/16	94	13	0.27		
21. p-Hydroxy phenylamino	100	Oral	11/12	92	14/16	88				
22. p-Methylphenylamino	100	Oral	10/12	83	14/16	88	5			
23. o-Carboxyphenylamino	100	Oral	11/12	92	14/16	88				
24. p-Aminophenyl	50	I.P.	6/12	50	16/20	80	30	>0.05 <0.10		

was highest with the alkyloxytriazines containing three or four carbon atoms in the aliphatic chain. Higher homologues, pentoxy to nonoxy, were less active; solubility was diminished. Incorporation of a tertiary amine in the aliphatic chain resulted in inactive compounds, i.e., dimethylamino- and B-morpholinoethoxy diaminotriazines (nos. 14 and 16). Of the two aryloxy derivatives (nos. 17 and 18), only the phenoxy compound exhibited some activity. It is of interest to note that melamine itself, and its derivatives devoid of an ether linkage, were found to be ineffective anti-histamine agents. Thus, it is apparent that anti-histamine activity within this series of compounds is related to the ether linkage.

Three alkyloxytriazines were also administered orally. Activity was demonstrated with the ethoxy and isopropoxy compounds, whereas the propoxy homologue failed to exhibit a significant effect with the dose employed. The oral doses were only twice the minimal effective dose given intraperitoneally, but nevertheless were effective in two of three cases.

B. *Acute toxicity.* The acute toxicity of the propoxy and butoxy compounds which were the most active (nos. 3, 4, 6, and 7) is nearly one-half that of aminophylline. Lower homologues possess low toxicity and activity, whereas higher homologues are more toxic and exhibit low activity, if any. The ratio of LD-50 in mice to minimal effective dose in guinea pigs ranges from 25 to 40 for the most effective triazines, as compared with only 5 for aminophylline. Symptoms produced in mice by alkyloxytriazines during the course of LD-50 determinations included ataxia, depression, and vasodilatation. Large but non-fatal doses produced hypnosis or depression which obtained for periods of 15 to 30 minutes in mice. Butoxy and propoxy triazines were injected intravenously in rabbits and produced discernible dilatation of ear vessels but no depression or hypnosis (or deaths) following doses of 25 to 200 mgm./kgm. administered over a period of 3 minutes.

TABLE 2

Maximal dilutions of drugs which inhibit the spasmogenic effects of histamine, barium, and acetylcholine on isolated guinea pig ileum

DRUG	HISTAMINE DIPHOSPHATE	BARIUM CHLORIDE	ACETYLCHOLINE BROMIDE	ANTI-ASTHMATIC INDEX†
Theophylline-ethylenediamine (Aminophylline)	1: 700- 1,000	1: 333- 666	1: 333- 666	1
1. 2-Sec.-n-Butoxy-4,6-diamino-s-triazine*	1:5,000-10,000	1:3,333-5,000	1:3,333-5,000	4
2. 2-iso-Propoxy-4,6-diamino-s-triazine	1:6,000-10,000	1:1,250-2,500	1:1,666-2,500	4
3. 2-n-Propoxy-4,6-diamino-s-triazine	1:1,000- 2,000	1:2,000-3,333	1:1,666-2,500	4

* All triazines as hydrochlorides; arranged in order of decreasing potency.

† Potency relative to alleviation of histamine-induced asthma; see table 1.

C. *Intestinal muscle.* Data contained in table 2 represent dilutions of aminophylline and three alkyloxytriazines which were capable of inhibiting the spasmogenic action of histamine, barium, and acetylcholine. The triazines are tabulated in order of decreasing potency. A typical experiment is presented in fig. 1.

Aminophylline was capable of preventing spasm but exhibited no definite preferential antagonism to any individual spasmogenic agent. Emmelin *et al.* (9) have reported similar results. Whereas the three alkyloxytriazines chosen for further study were each four times as effective as aminophylline in histamine-induced asthma, the isopropoxy- and secondary butoxy-diaminotriazines proved to be ten times more effective than aminophylline in antagonizing the action of histamine on intestinal muscle. The butoxy compound exhibited the same superiority over aminophylline with respect to barium and acetylcholine antagonism and showed no remarkable preferential action against any of the three spasmogenic agents. The isopropoxy compound was appreciably less effective against barium and acetylcholine than against histamine. The third compound, 2-n-propoxy-4,6-diamino-*s*-triazine, was two to five times more active than aminophylline against the various spasmogenic agents. There was no definite evidence of preferential action.

In a few of the experiments it was noted that slight, transient spasm of the muscle occurred when the drugs were added to the bath. This effect was not correlatable with various degrees of acidity of the triazines. Furthermore, aminophylline, which is alkaline, sometimes had the same effect and is known to be somewhat irritating when administered to animals (10). This transient initial spasm of isolated muscle has been reported (9) with dilutions of aminophylline similar to those which were employed in these experiments.

DISCUSSION. Among the twenty-four compounds in a series of 2-substituted diaminotriazines there were nineteen which contained an ether linkage and the majority of these compounds exhibited the ability to prevent histamine-induced bronchoconstriction in guinea pigs. The quantitative data clearly indicate that several of these ethers are four times more effective than aminophylline. No anti-histamine effectiveness was encountered in those compounds which did not possess an ether linkage.

It is concluded from the studies with guinea pig ileum that 2-sec.-butoxy-4,6-diamino-*s*-triazine is demonstrably superior to aminophylline and to propoxy- and iso-propoxy-diaminotriazines relative to antagonism of histamine, barium, and acetylcholines. No difference in activity among these triazines was demonstrated in the experiments dealing with prevention of bronchoconstriction in guinea pigs exposed to atomized histamine. The degree and order of activity would probably vary if studies were made on other types of smooth muscle.

Since only a few types of synthetic compounds are known to be comparatively potent histamine antagonists it is of interest to note their chemical structure. Among the compounds which do not contain oxygen in an ether linkage but which possess anti-histamine properties are isonipecaine (Demerol) (11, 12) and N-phenyl-N-ethyl-N′-diethylethylene diamine (1571F) (1, 3, 13, 14), as well as several derivatives of the latter (15–22). From the series of phenolic ethers synthesized by Fourneau, 2-isopropyl-5-methylphenoxyethyldiethylamine (929F) was found to have the strongest anti-histamine action (1). The activity of these phenolic ethers prompted a study (3, 4) to determine whether benzhydryl alkamine ethers possessed similar properties. Several compounds of this latter series were found to be comparatively strong anti-histamine agents. Benadryl (B-dimethylaminoethyl benzhydryl ether hydrochloride) relieves histamine-induced bronchoconstriction and anaphylactic shock in guinea pigs (3, 4) and also partially prevents the depressor action of histamine (5).

Thus, phenolic ethers, benzhydryl ethers and diaminotriazine ethers all possess anti-histamine activity. Although the triazines are least potent, their activity is greater than antispasmodics such as aminophylline, papaverine, and atropine (12) with respect to prevention of histamine-induced bronchoconstriction in guinea pigs. Further pharmacological studies will be necessary to determine the effectiveness of alkyloxytriazines as anti-histamine and general antispasmodic agents on smooth muscle in structures other than the bronchioles and intestines.

SUMMARY

Among a series of twenty-four 2-substituted diaminotriazines, several (propoxy, isopropoxy, isobutoxy, sec.-butoxy and cyclohexoxy) were found to be four times as efficacious as aminophylline in preventing experimental asthma induced in guinea pigs with atomized histamine. These triazine ethers are less potent than phenolic ethers and benzhydryl ethers with respect to anti-histamine action as tested on bronchiolar smooth muscle. The ratio of LD-50 in mice to minimal effective dose in guinea pigs was 25 to 40 for alkyloxydiaminotriazines, as compared with only 5 for aminophylline.

Alkyloxytriazines prevented spasm of guinea pig intestinal muscle which ordinarily follows addition of histamine, barium, or acetylcholine to the muscle bath. The anti-histamine action of three triazines tested on intestinal tissue was two to ten times that of aminophylline. These compounds are not specific histamine antagonists for the spasmogenic effects of barium and acetylcholine were also antagonized by dilutions of drug comparable to those effective against histamine.

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THE CUTANEOUS VASODILATING ACTION OF PITRESSIN[1]

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It is well known that Pitressin raises the blood pressure by peripheral constriction and influences the myocardium through coronary constriction. Though it can cause a fall in blood pressure from myocardial weakening in experimental animals, no direct mention is made in the literature of any vasodilating action of Pitressin.

Geiling and DeLawder (1) noticed that the femoral venous blood in the unanesthetized dog became more arterial in appearance following Pitressin. They studied the blood gas and lactic acid changes in the venous blood in dogs. With intravenous doses of Pitressin (10 to 12 units/animal) the oxygen content was increased and the carbon dioxide content was decreased for a period of from 5 to 10 minutes. *This effect will be referred to as the arterial shift produced by Pitressin.* Following this arterial shift, the conditions were reversed for various periods of time up to one hour. *This will be referred to as the venous shift.* During both shifts, the lactic acid content was always increased. These authors noted that only the venous shift occurred in the external jugular vein. In later papers from Geiling laboratory (2) it was observed that the oxygen consumption was decreased during the arterial shift and increased during the venous shift. These observations led to the suggestion that Pitressin might produce these effects by inhibiting certain enzymes in the tissues in a manner resembling the effect of cyanide or that the blood gas changes and lactic acid increase could have been produced by cutaneous constriction and simultaneous muscular vasodilation (1, 2). However, the decreased oxygen consumption could not be explained in the latter manner so the inhibition of oxidative processes has seemed to be the probable explanation (3, 4).

As a result of two observations in this laboratory, the problem has been reinvestigated. These observations were first, that some patients experience a sensation of surface warmth following intravenous administration of Pitressin and second, that pulse contours from two of about seventy cats have shown the presence of vasodilation following Pitressin (see fig. 1).

GENERAL METHODS. All of the animals were narcotized with 5 mgm./kgm. of morphine. When extensive operative procedures were necessary, ether was used as the anesthetic. Otherwise, the femoral artery and vein were exposed under procaine local anesthesia.

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[1] Appreciation is expressed to Lederle Laboratories for supplying the heparin used in the flowmeter studies.

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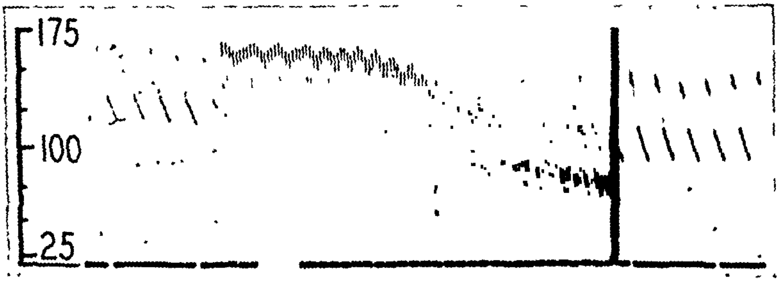

Fig. 1. Femoral Pressure Pulses from one of Seventy Cats Studied

Only two have shown this type of record. This cat received 150 mgm./kgm. of urethane three hours before these tracings were recorded. The other cat had received only procaine HCl locally in order to expose the femoral vessels. In both cases tachyphylaxis to the depressor effect evidently developed since a second injection of Pitressin elevated the arterial pressure. At signal, 1 unit/kgm. of Pitressin was injected intravenously. Tracings, recorded on fast moving paper 1 minute before and 1 minute after the injection are inserted at each side of the figure. These inserts show the details of pressure pulses during the control period and shortly after the maximal reduction in blood pressure. Notice the fall in arterial systolic and diastolic pressure which is atypical of Pitressin. Definite vasodilation apparently is present since for any given pressure, the rate of pressure descent during diastole, is markedly increased after the injection of Pitressin. At any given pressure an increase in the rate of pressure descent during diastole, indicates vasodilation (14).

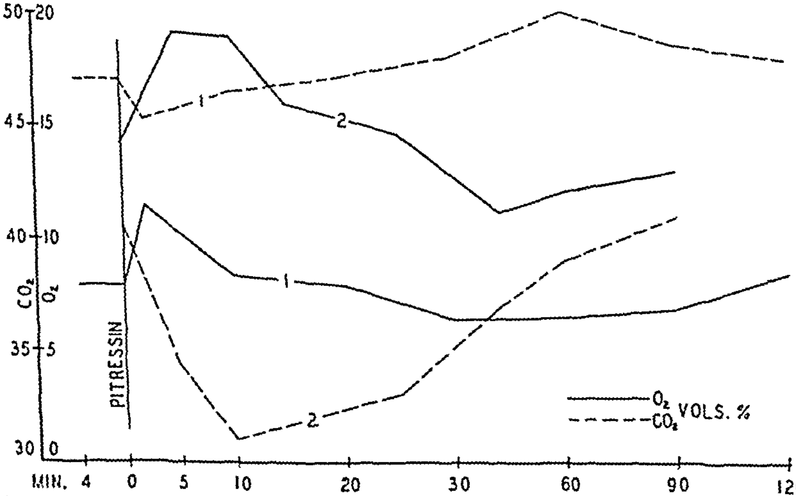

Fig. 2. Oxygen and Carbon Dioxide Content of Femoral Venous Blood of Dog

Curves one = results with intravenous injection of 0.1 unit/kgm. of Pitressin. Curves two = results of Geiling and DeLawder (2) with 0.5 unit/kgm. of Pitressin intravenously.

Venous and arterial blood samples were drawn into oxalated, mercury-filled syringes similar to those described by Adriani (5). Oxygen and carbon dioxide were determined manometrically by the method of Van Slyke (6). Values for arterial gas concentrations are omitted from this paper since the oxygen and carbon dioxide values for arterial blood showed no significant change during any experiment. Consistently typical arterial and venous shifts of the femoral venous blood were obtained with 0.1 unit/kgm. of Pitressin. As shown by fig. 2, the changes, though less marked and less prolonged, did not differ qualitatively from those produced by 0.5 unit/kgm. (approximate dose used by Geiling and DeLawder (2)).

Seven general approaches were made to the problem. The special procedures used in each group will be described along with the results obtained. In most but not all of any group of experiments, control blood gas responses to Pitressin were obtained 1 to 5 days before the experiment.

RESULTS AND DISCUSSION. 1. *Skinless leg.* In five etherized dogs, the skin was completely removed from one leg, the femoral artery and vein were exposed in both legs and the blood pressure recorded from the carotid artery with a mercury manometer. In two of the dogs, a Soskin type bubble-flowmeter (7) was inserted into each femoral artery and coagulation of blood was prevented by administration of heparin. In the other three dogs, simultaneously venous samples were taken from each leg together with arterial control samples.

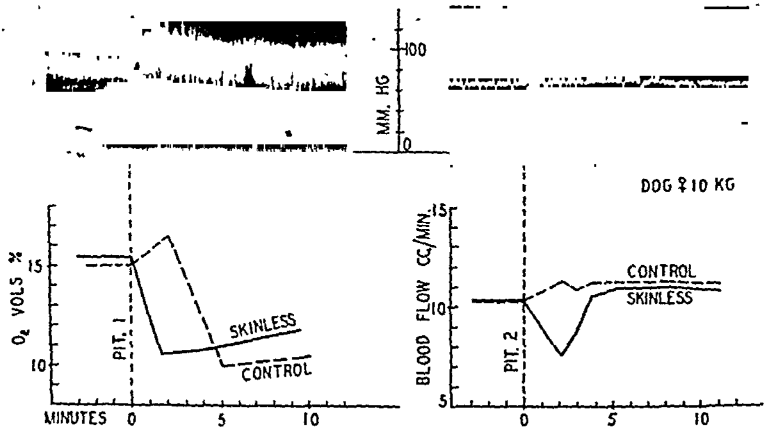

FIG. 3. MEAN ARTERIAL BLOOD PRESSURE, OXYGEN CONTENT AND BLOOD FLOW TO SKINLESS AND CONTROL LEGS OF MORPHINIZED ETHERIZED DOG

Pit 1 = Intravenous injection of 0.2 unit/kgm. of Pitressin. Pit 2 = Intra-arterial injection simultaneously in both legs of 0 02 unit/leg.

A 2 unit dose of Pitressin, administered intravenously, fig. 3—Pit. 1, produced the characteristic arterial shift in the control leg followed by the venous shift while the skinless leg showed only the venous shift. The absence of the arterial shift in the skinless leg constitutes strong proof that the arterial shift in the control leg was not caused by inhibition of enzymes. It seems more likely that the arterial shift must be vascular in origin. It could arise from an increase in blood flow through the skin, either passively from a rise in arterial pressure from constriction of vessels of other areas including muscles, see fig. 3—Pit. 1, or actively from vasodilation. A part of the increase in blood flow must originate from vasodilation since flow increased even though there is no elevation in arterial pressure in some animals, see fig. 3—Pit. 2.

The sequence of events that occurs may be as follows. The Pitressin constricts most of the peripheral blood vessels, especially those of the muscles, which as previously shown (8), decreases total blood flow in the leg. The blood which is now returned in the femoral vein is mostly from passively and actively dilated skin vessels (probably A-V shunts) and is more arterial in nature. Subsequently, as vasoconstriction of muscular areas diminishes, blood flow from these muscular areas is proportionally increased. Since it is markedly venous in character, the femoral venous blood now shows the venous shift.

2. *Coronary blood samples.* The heart of a dog was exposed under ether anesthesia and artificial respiration. Coronary blood samples were taken by inserting a small needle into one of the large coronary veins. The coronary venous blood shows only the venous shift after Pitressin. In this organ, almost entirely muscular in nature, no evidence of enzyme inhibition was obtained.

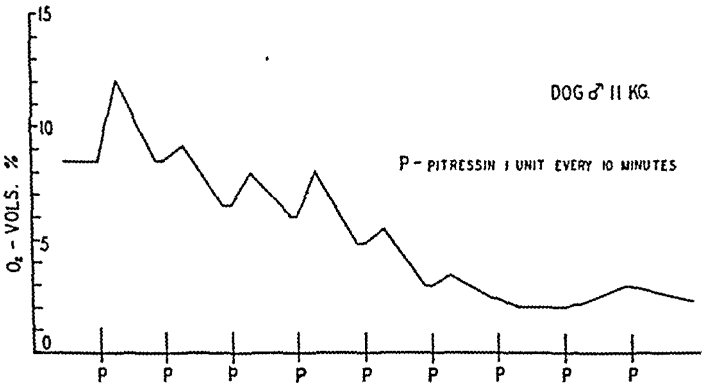

FIG. 4. OXYGEN CONTENT OF FEMORAL VENOUS BLOOD P = 1 UNIT OF PITRESSIN/DOG INTRAVENOUSLY

3. *Repeated doses of Pitressin.* One unit of Pitressin was administered intravenously to a narcotized dog every ten minutes. Femoral venous samples were taken one minute before and three minutes after each dose of Pitressin. Results from one of these animals are illustrated by fig. 4. The arterial shift occurred with each of the first six doses; thereafter the arterial shift did not occur. The venous shift became greater and greater with each dose. The same total amount of Pitressin administered in one injection (fig. 2) produced an arterial shift as well as the venous shift. The development of tachyphylaxis is rather strong evidence that reduction in oxygen consumption from enzyme inhibition is not responsible for the arterial shift. These results show that the arterial shift is not due to splenic contraction. The first dose of Pitressin should cause full splenic contraction and there is not time between doses for splenic relaxation (9).

4. *Effect of epinephrine.* Two types of procedures were used to test the influence of epinephrine on the Pitressin arterial shift. In one case, the epinephrine was given intravenously just after the Pitressin. In the other, the epinephrine was given intra-arterially into the test leg just before the Pitressin,

see fig. 5. Epinephrine in either case removed the arterial shift and intensified the venous shift. Epinephrine is known to constrict cutaneous vessels (10). Consequently it would be expected to interfere with the arterial shift, and it does. This is additional evidence that the arterial shift after Pitressin arises from cutaneous vascular changes.

Though Pitressin and epinephrine elevate the arterial pressure, they generally have opposite effects on any particular vascular area. Vessels to the heart and skeletal muscles are constricted by Pitressin and dilated by epinephrine. Cutaneous vessels are dilated by Pitressin and constricted by epinephrine. Since the vasoconstrictor action of both drugs predominate, these antagonistic drugs have synergistic effects upon the arterial pressure.

5. *Effect of denervation.* One leg of the dog was denervated under morphine narcosis and procaine anesthesia; the femoral and sciatic nerves being cut high in the leg. The other leg was kept as a control. In the freshly denervated leg, the Pitressin arterial and venous shifts were both present, see fig. 6. The denervation, however, caused the venous blood to be much more arterial before the Pitressin was administered.

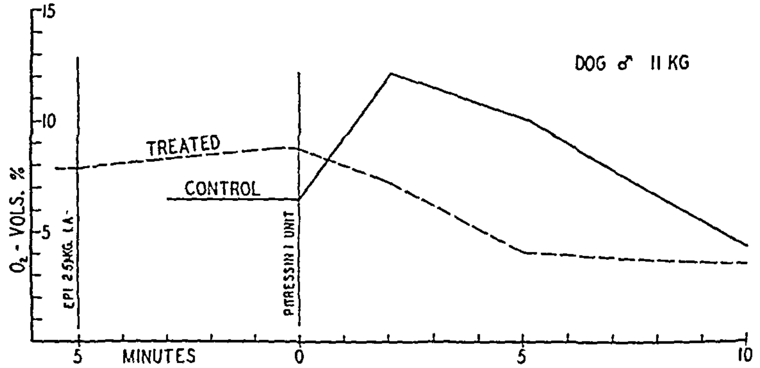

FIG. 5. OXYGEN CONTENT OF FEMORAL VENOUS BLOOD

Curve labeled "treated" was obtained from data from leg where 2.5 gamma/kgm. of epinephrine was injected into the femoral artery five minutes before the intravenous injection of 1 unit of Pitressin/dog.

Eight days after denervation, see fig. 7, the late effect of denervation is to abolish the arterial shift and to intensify the venous shift. Two possible explanations suggest themselves. Nerve degeneration may cause greater sensitivity of the peripheral vasoconstrictor mechanism to Pitressin or Pitressin may cause the liberation of epinephrine. This latter possibility is evidently eliminated by the observation that 0.2 unit of Pitressin injected into the artery caused complete arrest of venous outflow from the leg where nerve degeneration of 60 days duration was present. This small dose of Pitressin into the artery arrested blood flow from the leg before appreciable amounts of the drug could ever have reached the adrenals. Nerve degeneration, as is the case with epinephrine (11), apparently increases the susceptibility of the constrictors to Pitressin, since intra-arterial injection of this dose into the control leg did not completely arrest the flow and did produce an initial arterial shift followed by a venous shift.

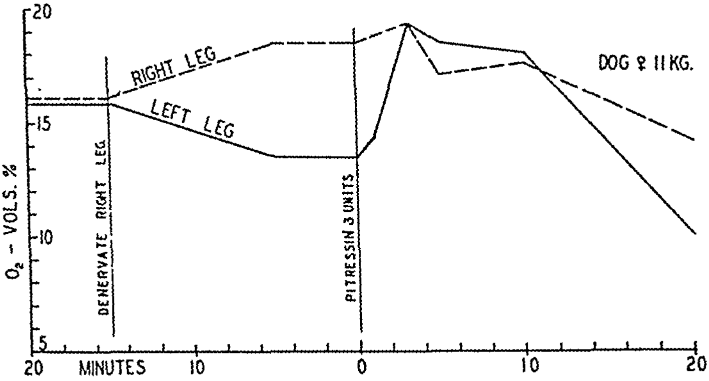

FIG. 6. OXYGEN CONTENT OF FEMORAL VENOUS BLOOD

Right leg was denervated fifteen minutes before the intravenous injection of 3 units of Pitressin/dog.

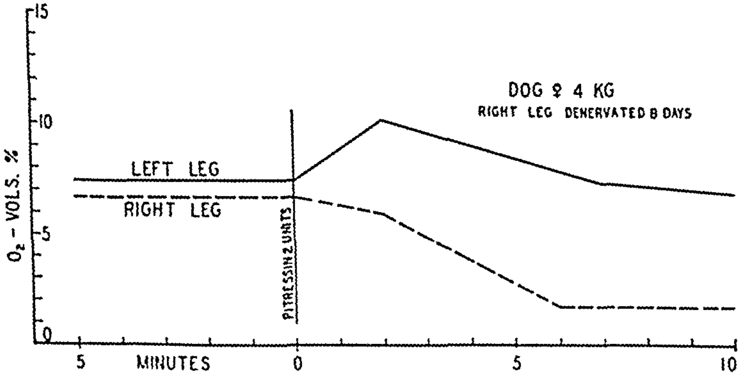

FIG. 7. OXYGEN CONTENT OF FEMORAL VENOUS BLOOD

Right leg was denervated eight days before the injection of Pitressin

This observation that Pitressin completely arrests blood flow in the denervated leg indicates also that the drug is capable potentially of causing both dilation and constriction of skin vessels.

6. *Rabbit.* It might be expected that these two actions might vary quantitatively in different species. The study was extended to include the rabbit. Under morphine narcosis and procaine anesthesia, the trachea of rabbits were exposed and connected by a small cannula to a Sanborn basal metabolism appa-

ratus. The femoral artery and vein were also exposed. Figure 8 illustrates the usual effect of Pitressin on the oxygen consumption and on the femoral venous blood gas concentrations. The rabbit does not show the arterial shift but does show the decreased oxygen consumption described by Geiling and DeLawder (2). The absence of the arterial shift in rabbits is in agreement with the observation that Pitressin causes constriction of ear vessels of rabbits (12).

The main fact from which Geiling et al derived their theory that Pitressin inhibited oxidative processes was that the oxygen consumption of the animal decreases during the period of the arterial shift. This indicates an anaerobic type of metabolism. However, in the rabbit, the arterial shift does not occur, yet the decreased oxygen consumption is still present. Therefore, the decrease in oxygen consumption is probably not directly related to the arterial shift and can be explained as follows: Since cardiac output is markedly reduced (13), oxygen consumption by this organ is decreased. In addition, reduced oxygen consumption is possibly due to the vasoconstrictive action of Pitressin in certain organs that utilize large amounts of oxygen. The constricion results in poor blood flow and in a decreased uptake of oxygen. Possible sites of this vasoconstriction are skeletal muscles, cardiac muscle (13), viscera (9), and kidney (8).

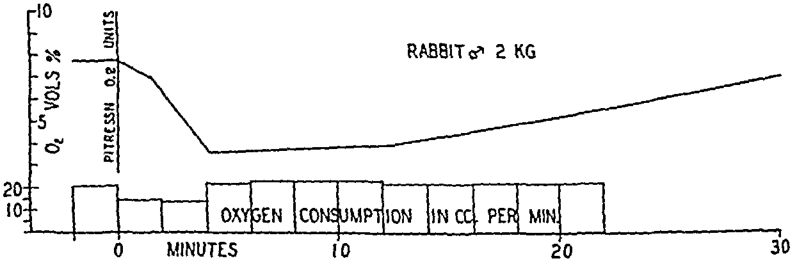

Fig. 8. Oxygen Content of Femoral Venous Blood and Oxygen Consumption of Rabbit
At "0" minute 0.1 unit/kgm. of Pitressin was injected intravenously

CONCLUSIONS

In dogs, intravenous injections of Pitressin transiently increase blood flow through cutaneous vessels and decrease blood flow through vessels of muscular areas. The increased cutaneous flow originates passively from elevation of arterial pressure and actively from dilation of cutaneous vessels.

Nerve section does not abolish these effects. The amount of cutaneous vasodilation varies in different dogs, varies under different conditions, and varies in different species. It is absent in most rabbits. In dogs it is abolished by preinjection of epinephrine and by nerve degeneration. Tachyphylaxis develops to this vasodilator action of Pitressin. Pitressin also possesses cutaneous vasoconstrictive activity in rabbits and to a lesser extent in dogs. Nerve degeneration heightens the sensitivity of blood vessels to the vasoconstrictive action of Pitressin.

Through vasoconstriction, Pitressin restricts the amount of oxygen available in tissue and thereby limits oxidative processes. No evidence was found that Pitressin directly inhibits oxidative processes.

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PHARMACOLOGICAL STUDIES ON STREPTOTHRICIN

HARRY J. ROBINSON, OTTO E. GRAESSLE, MARY GUNDEL AND ROBERT H. SILBER

From the Merck Institute for Therapeutic Research, Rahway, New Jersey

Received for publication August 27, 1945

INTRODUCTION. Reports from this laboratory indicated that streptothricin was an effective chemotherapeutic agent for the treatment of experimental infections produced by gram-negative bacteria. Attention was drawn to the possible value of this drug in bacillary dysentery, typhoid fever, brucellosis and in the local treatment of infected wounds and burns (1, 2, 3). However, crude streptothricin was shown to produce certain toxic effects in mice and it was stated that the ultimate value of streptothricin as a chemotherapeutic agent for the systemic treatment of bacterial infections in man would largely depend upon the toxicity of the purified product. To date, the pharmacological properties of streptothricin have not been described in the literature. Certain phases of this problem are considered in this communication and show that in its present form, streptothricin appears to be too toxic for systemic use in man.

MATERIALS. The source and care of the animals used in this study have been described in a previous report (4) and therefore will not be considered here.

The streptothricin[1] used was obtained from two different types of broth and varied in potency from 50,000 to 500,000 units per gram. The first preparation, which for convenience henceforth shall be called *streptothricin W*, was isolated from a tryptone broth described by Waksman and Woodruff in 1942 (5). The second preparation, which will be designated as *streptothricin F*, was obtained by growing the same organism in a corn steep liquor medium developed by Foster and Woodruff in 1943 (6). All preparations of streptothricin were readily soluble in water. Aqueous solutions of the drug were acidic, but could be readily neutralized by the addition of small amounts of sodium hydroxide.

METHODS. Toxicity tests were performed in a variety of animal species by giving the drug parenterally and by mouth. Since a number of deaths occurred 5 to 8 days after a single dose of streptothricin was given, observations were extended over a 10 to 20 day period. In the chronic toxicity studies in mice, rats and dogs, the food and water consumption, as well as the weight and general appearance of the animals were recorded. In the pharmacological studies, the blood pressure was measured by means of a mercury manometer connected to the carotid artery, and volume changes in the intestine, liver, and hind leg were recorded by means of a plethysmograph. Respiration was recorded by means of a Tambour connected directly to a Y-tube inserted into the trachea, and the heart rate by the method of Kniazuk (7). The drug was injected directly into the femoral or jugular vein, as indicated in individual experiments. Studies on the isolated ear were performed according to the method of Rischbieter (8). The influence of these drugs on the isolated rabbit intestine and uterus was determined by suspending an intestinal or uterine strip in a 100 cc. bath of oxygenated Locke's solution at constant temperature, and adding the test drug to this bath. Water diuresis was measured as described in a previous communication (9).

In the biochemical studies, the following methods were used: the micro-Kjeldahl procedure for the determination of plasma total nitrogen and non-protein nitrogen (10% trichloracetic acid blood filtrate), the Conway diffusion method for blood urea (10), the Cullen and Van Slyke procedure for plasma fibrinogen (11), and the method of Lowry and Hastings for specific gravity of serum (12).

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[1] The streptothricin employed in these studies was obtained from the chemists of the Research Laboratories of Merck & Co., Inc., from cultures grown by Dr. J. W. Foster.

In the absorption and excretion studies the concentration of streptothricin was determined by means of a modification of the method of Foster and Woodruff (13). Details of this procedure have recently been described by Stebbins and Robinson (14).

RESULTS. *Acute toxicity.* A summary of the results obtained in the acute toxicity experiments with *streptothricin F and W* in mice, is presented in table 1. Similar findings were obtained in rats and dogs. These findings show that streptothricin obtained from the medium described by Foster and Woodruff (6) was somewhat more toxic than the streptothricin isolated from the original medium of Waksman (5). The difference in the toxicity of the two preparations was particularly striking if the observation period was limited to a 24 hour period. Furthermore, there was a marked difference in the nature of the acute toxic signs produced by the two preparations. With *streptothricin W*, a single intravenous or subcutaneous injection of a lethal dose produced no immediate untoward effects except a mild transitory blush reaction in dogs, such as that produced by the injection of small doses of histamine. There was no evidence of a temperature rise, indicating an absence of pyrogens. A number of days after the injection, the animals became lethargic and depressed, refused food and water, and died in coma between the 5th and 12th day, depending upon the purity of the preparation used. However, toxic signs could be produced immediately after drug administration if very large doses were injected. Other toxic signs noted 3–4 days before death were dehydration, a marked loss of body weight, and a decrease of several degrees in the body temperature. In mice, both the salivary and lacrimal glands were stimulated by the drug so that copious salivation and tears were noted simultaneously with the onset of the other toxic signs. These toxic signs were similar to those produced by the injection of albumoses or peptone (15). Upon autopsy[2] marked congestion of the liver was found with general dilation of the blood vessels throughout the viscera. The kidneys appeared blanched and the peritoneum edematous.

TABLE 1

Acute subcutaneous toxicity of streptothricin for mice (24 hr. observation)

NO. OF MICE	DOSE	STREPTOTHRICIN "F"			STREPTOTHRICIN "W"		
		Number dead	Number alive	Per cent dead	Number dead	Number alive	Per cent dead
	units per kgm.						
40	62,500	0	40	0	0	40	0
40	125,000	40	0	100	0	40	0
40	250,000	40	0	100	0	40	0
40	500,000	40	0	100	16	24	40

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[2] A complete study of the histopathology of streptothricin will be published elsewhere by Drs. C. W. Mushett and H. Martland.

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METHODS. Toxicity tests were performed in a variety of animal species by giving the drug parenterally and by mouth. Since a number of deaths occurred 5 to 8 days after a single dose of streptothricin was given, observations were extended over a 10 to 20 day period. In the chronic toxicity studies in mice, rats and dogs, the food and water consumption, as well as the weight and general appearance of the animals were recorded. In the pharmacological studies, the blood pressure was measured by means of a mercury manometer connected to the carotid artery, and volume changes in the intestine, liver, and hind leg were recorded by means of a plethysmograph. Respiration was recorded by means of a Tambour connected directly to a Y-tube inserted into the trachea, and the heart rate by the method of Kniazuk (7). The drug was injected directly into the femoral or jugular vein, as indicated in individual experiments. Studies on the isolated ear were performed according to the method of Rischbieter (8). The influence of these drugs on the isolated rabbit intestine and uterus was determined by suspending an intestinal or uterine strip in a 100 cc. bath of oxygenated Locke's solution at constant temperature, and adding the test drug to this bath. Water diuresis was measured as described in a previous communication (9).

In the biochemical studies, the following methods were used: the micro-Kjeldahl prodelayed toxic signs were identical with those produced by the streptothricin prior to its passage through the animal body.

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[1] The streptothricin employed in these studies was obtained from the chemists of the Research Laboratories of Merck & Co., Inc., from cultures grown by Dr. J. W. Foster.

Although the foregoing results strongly suggest that the toxicity of streptothricin is closely related to the active fraction, a final statement in this matter cannot be made until pure streptothricin is available for study.

Chronic toxicity. Mice and rats. Mice and rats injected subcutaneously with doses of *streptothricin W* ranging from 625 to 5000 units per kgm. daily over a period of one month tolerated the drug. Larger doses of 10,000 and 20,000 units per kgm. were lethal.

The toxic signs observed following the repeated injection of *streptothricin W* included a marked fall in body temperature and a loss of muscle tone and body weight. The loss of body weight appeared to be in part the result of a decreased intake of both food and water. Examination of the urine and urinary volume showed that rats receiving lethal doses developed gross hematuria, followed by oliguria and eventually anuria 24 to 48 hours before death. As expected, the N.P.N. of the blood was elevated considerably above normal and suggested that death was due to renal insufficiency. In addition, lethal doses of streptothricin given subcutaneously to rats increased the plasma fibrinogen level from 250–300 mg. % to 900–1200 mg. % indicating rather extensive tissue damage. Measurements of the serum specific gravity showed that the animals also became dehydrated before death, possibly the result of the decreased water intake. As will be described in detail in a later section of this report, the anuria resulting from the injection of streptothricin was not due solely to the decreased water intake, for a similar condition occurred even when large amounts of forced fluids were given.

A number of mice surviving longer than one month developed alopecia and showed signs of tremors and polyneuritis. Bacteriological examination of the intestinal flora of mice fed streptothricin revealed a marked decrease in the number of lactose-fermenting bacteria of the coli-form group, similar to that observed following the administration of certain sulfonamides (16). These findings suggested that streptothricin might have induced a vitamin deficiency through a modification of the intestinal flora. However, the foregoing deficiency signs were not alleviated by the injection of massive doses of thiamine, riboflavin, pyridoxine, pantothenic acid, biotin or vitamin K_1.

Dogs. The findings in dogs were in many respects similar to those recorded for other animal species (table 3). Death resulting from the repeated injection of lethal doses of *streptothricin W* appeared to be due to renal insufficiency. Animals injected daily over a seven day period with doses of 625 to 2,500 units per kgm. tolerated the drug during this time without showing marked toxic effects. However, the dogs on the dose level above 2,500 units per kgm. became depressed, showed signs of oliguria and died in coma 4 to 5 days after the final injection of the drug. Larger doses produced death more rapidly. Prior to death, the N.P.N. and blood urea values were elevated considerably above the normal value. In addition, the specific gravity of the serum was increased, indicating dehydration and hemoconcentration. The latter factor complicated the results of the hematological studies in these animals, in that the observed

In certain respects, the toxic signs initiated by *streptothricin F* were similar to those just described for *streptothricin W*. However, the blush reaction in dogs was much more pronounced, and the majority of deaths occurred within the first few hours after drug administration. Death appeared to be the result of circulatory collapse and respiratory failure.

Preliminary data suggest that upon further purification the factor or factors associated with the immediate toxic effects caused by *streptothricin F* could be removed and when this was done, the preparation produced delayed toxic reactions very similar to, or identical with, those caused by *streptothricin W*.

Relationship of activity to acute toxicity. With *streptothricin W*, this phase of the investigation was studied using a number of preparations varying in potency from 50 to 500 units per mgm. of solid. The results show that 125,000 units per kgm. of all preparations were lethal for 60 to 100% of the mice (table 2). Since as much as 2,500 mgm. per kgm. was necessary to produce death in the case of the low potency material and as little as 250 mgm. per kgm. had the same effect when a more purified preparation was used, the toxicity of streptothricin appears to be associated with the active principle rather than with the inactive constituents. Moreover, the nature of the delayed toxic signs produced by the low potency material was similar to those caused by the purer samples.

TABLE 2

Subcutaneous toxicity of various streptothricin preparations

DOSE	DOSE	POTENCY OF SAMPLES IN UNITS PER MGM.						INACTIVATED BY ACID	RECOVERED* FROM URINE	
		50	160	200	300	400	500		Oral preparation	Subcutaneous preparation
		Per cent mortality								
units per 20 grams	*units per kgm.*									
625	31,250	0								
1250	62,500	50	43	20	14	14	0	0	0	0
2500	125,000	87	75	100	64	67	60	0	40	40
5000	250,000		100	100	100	100	100	0	100	80

* Streptothricin given orally and subcutaneously to dogs and the active principle isolated from the urine. The toxicity of both preparations was then determined by subcutaneous injection in mice.

Further evidence indicating that the toxicity of streptothricin might be associated with the active fraction was obtained by determining the toxicity of streptothricin after the preparation was inactivated by chemical methods. When this was done, both the toxicity and the activity were removed so that a dose of 1,250 mgm. per kgm. equivalent to 250,000 units per kgm. before inactivation, produced no outward signs of toxicity.

It was of interest to note that the toxicity of streptothricin isolated from the urine of dogs following oral and parenteral administration of the drug was of the same order as that of unmetabolized streptothricin.[3] Both the acute and

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[3] This material was isolated by Dr. David M. Tennent of the Merck Institute for Therapeutic Research; the details of which will be reported elsewhere.

PHARMACOLOGY. The pharmacodynamic action demonstrated by *streptothricin F and W* appeared to be due to the presence of two toxic factors. The first, which was found in relatively large amounts in *streptothricin F* and to some extent in *streptothricin W*, caused marked acute changes in the cardiovascular and respiratory system of rats, rabbits, cats and dogs and in this respect appeared to be similar to histamine or certain albumoses (15). The second toxic factor appeared to be related to the active principle and induced certain pathological changes in the kidneys which led to renal insufficiency and death a number of days after the drug was excreted from the body. Death following the injection of *streptothricin W* appeared to be due primarily to the nephrotoxic factor.

Effects on the cardiovascular system. The effects upon this system seen after the intravenous injection of either streptothricin preparation were similar to those observed following the injection of histamine or a similar base. While the results varied somewhat from animal to animal, the intensity of the effects produced with each preparation appeared to be proportional to the dose and the speed of the injection. In most rats and cats, a dose of 200 units per kgm. of *streptothricin F*, and 50,000 per kgm. of *streptothricin W*, caused a precipitous fall of blood pressure which was accompanied by, or in some cases followed by, a significant slowing of the heart rate. This bradycardia and the low blood pressure gradually returned to normal over a period of 10 minutes (figure 1). In rabbits, the findings were somewhat erratic and varied from animal to animal. In some cases the blood pressure increased, in some it decreased, while in other rabbits there was no effect at all. Histamine showed the same effect in this series of rabbits and suggested that these preparations might contain histamine.

Plethysmographic studies performed to measure the changes in the volume of the leg, kidney and intestine showed that the fall in blood pressure caused by these drugs was due largely to a vasodilation of the peripheral vascular system. On the other hand, the vessels of the kidney were constricted. These effects were transitory and the vascular system returned to normal within 10 to 15 minutes.

The results of these studies suggested the possibility that the acute pharmacological effects of *streptothricin F and W* might be due to the presence of histamine since it is well known that this substance is produced by microorganisms. The fact that none of the acute effects caused by streptothricin were blocked by the injection of atropine or vagotomy gives further support to this view. Central stimulation with caffeine or metrazole was found to overcome the foregoing depressing effects of *streptothricin F and W*.

In order to determine whether the foregoing effects were due to the presence of histamine, an attempt was made to destroy the blood pressure depressing factor by histaminase, an enzyme known to readily inactivate histamine. Streptothricin and excessive amounts of histaminase were mixed *in vitro* at 37°C. for 18 and 48 hours before animal inoculation. Control experiments showed that under these conditions this preparation of histaminase inactivated histamine and that the quantity added to streptothricin would have been increase in red blood cells as well as the hemoglobin concentration was possibly a result of hemoconcentration.

Monkeys. Two monkeys injected intramuscularly three times each day over a five day period with total daily doses of 20,000 units per kgm. of *streptothricin W* died 7 to 9 days after the last dose was given. The toxic signs were similar to those observed in other animal species. Two monkeys fed daily doses of 10,000 units per kgm. over a period of two months showed no outward signs of toxicity.

TABLE 3

Toxicity of streptothricin

DOG NUMBER	DOSE	DURATION OF TREATMENT	TOTAL DOSE	METHOD OF ADMINISTRATION	REMARKS
			Dogs		
	units per kgm. per day	*days*	*units*		
501	40,000	1	300,000	Intravenous	Dead in 12 days
497	50,000	1	700,000	Intravenous	Dead in 2 days
498	100,000	1	1,300,000	Intravenous	Dead in 2 days
449	625	7	63,437	Intravenous	Survived
459	1,250	7	113,750	Intravenous	Survived
495	2,500	7	323,750	Intravenous	Survived
299	5,000	7	700,000	Intravenous	Dead in 10 days
450	10,000	6	1,050,000	Intravenous	Dead in 9 days
504	10,000	9	787,000	Intravenous	Dead in 13 days
502	20,000	8	1,058,000	Intravenous	Dead in 9 days
503	40,000	4	1,048,000	Intravenous	Dead in 6 days
499	16,000	20	5,120,000	Oral	Dead in 20 days
496	25,000	7	2,625,000	Oral	Survived
493	36,000	7	4,032,000	Oral	Dead in 8 days
461	50,000	7	4,340,000	Oral	Dead in 8 days
			Monkeys		
MONKEY NUMBER					
83	5,000	5	92,400	Intramuscular	Sacrificed on 23rd day
80	5,000	5	87,500	Intramuscular	Sacrificed on 23rd day
24	11,500	60	1,800,000	Oral	Sacrificed on 61st day
37	10,000	60	1,800,000	Oral	Sacrificed on 61st day
77	20,000	5	250,000	Intramuscular	Dead on 14th day
79	20,000	5	350,000	Intramuscular	Dead on 12th day

Guinea pigs. The experiments with this species, although somewhat limited, showed that guinea pigs tolerated daily doses of 1000 units per kgm. of *streptothricin W* subcutaneously over a period of one month without signs of toxicity. Doses of 5000 units per kgm. were lethal.

spiratory failure (figure 1). When the drug was injected rapidly the depressing effect upon the respiration was markedly enhanced. The bradycardia and fall in blood pressure described in the foregoing section accompanied the decreased respiratory rate and depth. However, the respiratory effect was frequently produced without alterations in the cardiovascular system and therefore does not appear to be a secondary effect caused by changes in the circulatory system. Similar effects were produced by *streptothricin W* when ten to twenty times the therapeutic dose was injected.

Kidney function. The influence of streptothricin F and W upon kidney function was studied in rats by means of normal and water diuresis experiments and by measuring the blood urea and N.P.N. of the animals before and after drug administration.

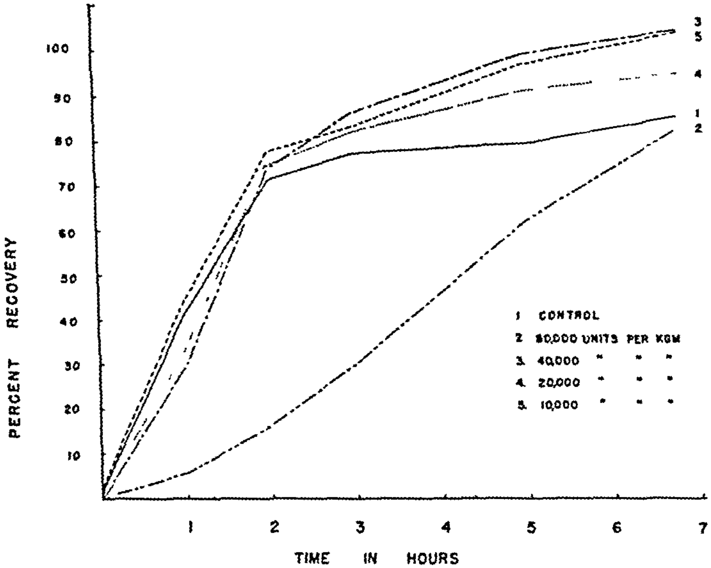

FIG. 2. ACUTE EFFECT OF STREPTOTHRICIN UPON THE WATER DIURESIS OF RATS
The effect of a single subcutaneous dose of streptothricin (potency 300 units per mgm.) on the excretion of water over a seven hour period.

Diuresis experiments. The results of the normal and water diuresis experiments are presented in figures 2, 3 and 4 and show that single large doses (80,000 units per kgm.) or multiple small daily doses of 10,000 units per kgm. suppressed and eventually completely inhibited the excretion of urine. These effects occurred with both streptothricin preparations and appeared to be a function of the amount of the drug administered. It will be noted that the effects of streptothricin upon the water diuresis appears to be two-fold. The first effect resulting from the subcutaneous administration of single large doses (80,000 units per kgm.) was a transitory suppression of the urinary output which subsufficient to inactivate all the histamine that might have been present. The results show that in spite of the 18 hour treatment the pharmacodynamic properties of streptothricin remained unchanged. However, when the mixture was incubated over the 48 hour period some of the histamine-like action of streptothricin was lost. These findings suggest that histamine is not the factor causing the foregoing pharmacological effects although the substance may be closely related to histamine.

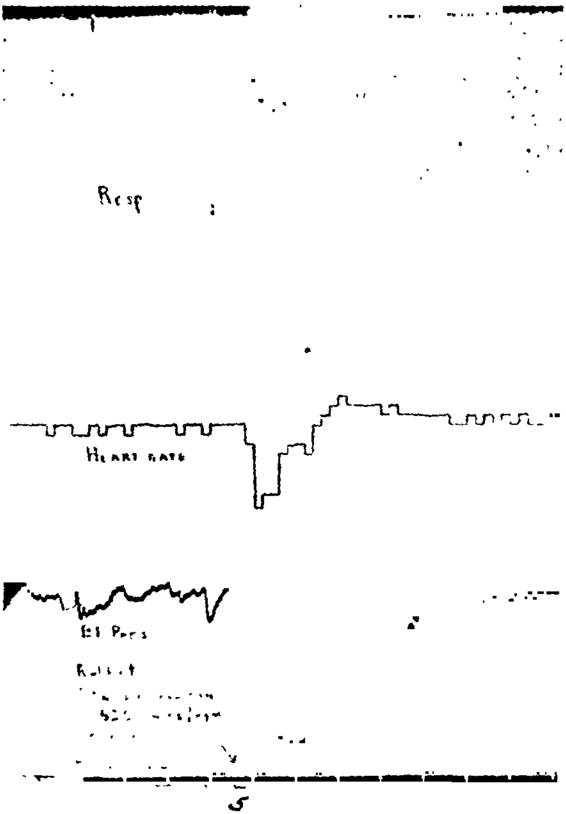

FIG. 1. INFLUENCE OF STREPTOTHRICIN ON THE HEART RATE, BLOOD PRESSURE AND RESPIRATION OF RABBITS

Respiratory system. The effect of streptothricin upon the respiratory system appeared to be a function of the dose and the rate of the injection. Thus the intravenous injection of therapeutic doses of *streptothricin F* was followed by an increase in both the rate and depth of respiration. Larger doses caused a decrease in both the respiratory rate and depth, and in some cases caused reanuria always occurred 24 to 48 hours before death (figures 3 and 4). The anuria could not be prevented or overcome by the prophylactic administration of various diuretics including urea and theophylline. Likewise, daily doses of desoxycorticosterone had no apparent influence on the condition. This hormone was tried in view of the nature of the toxic signs produced by *streptothricin W* which in certain respects presented a picture similar to that resulting from adrenal cortical deficiency.

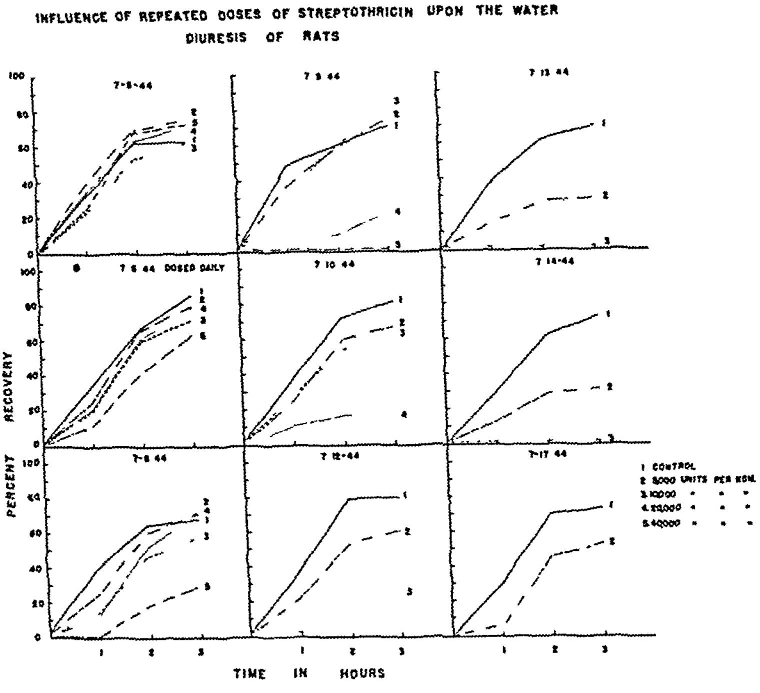

FIG. 4. INFLUENCE OF REPEATED DOSES OF STREPTOTHRICIN UPON THE WATER DIURESIS OF RATS

Rats treated subcutaneously with streptothricin (potency 300 units per mgm). Treatment began on 7-6-44 and was given daily until 7-17-44 (5 rats per group).

The biochemical findings in this series of rats were similar to those described for other animal species earlier in this communication. Blood urea and N.P.N. values increased rapidly following the suppression of the urinary output and reached a peak just before death.

Local application of streptothricin. Rabbit eye. Neutral aqueous solutions of *streptothricin F or W*, containing 200 units per cc. or more, applied to the eye over a period of 30 minutes produced inflammation. The degree and course of the reaction was a function of the drug concentration and the duration of contact with the eye. In general, the reaction was characterized by a delayed sided within 7 to 8 hours after drug administration. This suppression was most likely the result of the vasoconstriction observed in the renal vessels following the injection of *streptothricin F or W*. The second effect, which occurred 2–3 days after a single injection of 80,000 units per kgm. of the drug was

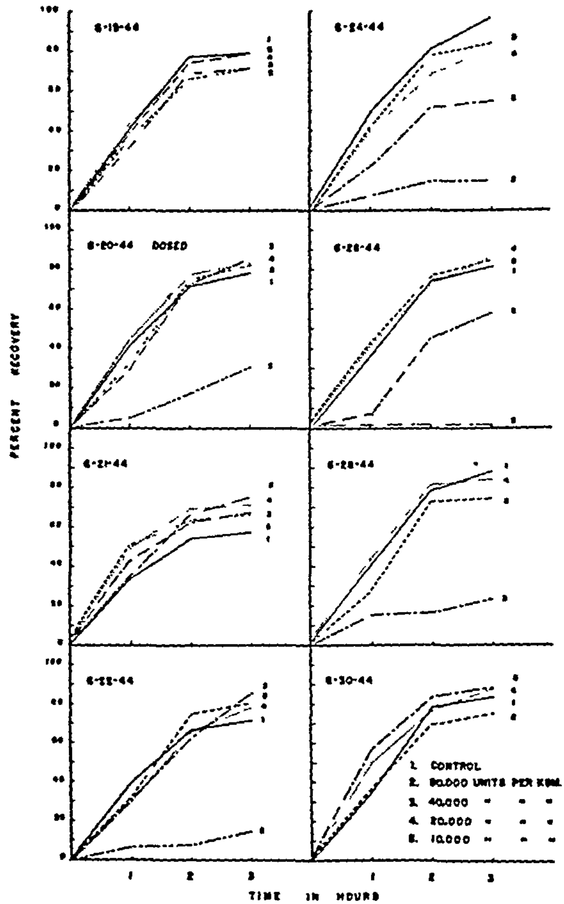

FIG. 3. DELAYED EFFECT OF STREPTOTHRICIN ON WATER DIURESES OF RATS

Findings show the gradual development of renal insufficiency following the injection of a single large dose of streptothricin (potency 300 units per mgm.) injected subcutaneously on 6-20-44 and the water diuresis measured daily thereafter until 6-30-44.

characterized by the eventual complete suppression of both the normal and the water diuresis. Similar results were obtained following the subcutaneous injection of doses of 10,000 to 40,000 units per kgm. given daily over a 3 to 30 day period depending on the dose administered. It will be observed that intervals. Thus, dogs injected intramuscularly with daily doses of 15,000 units per kgm. given in the form of divided doses every 3 hours, showed blood concentrations between 2–5 units per cc. Examination of the bile showed that approximately 10% of the streptothricin could be accounted for by excretion into the duodenum. When the drug was administered orally, much of it could be found in the stools and low blood concentrations were obtained.

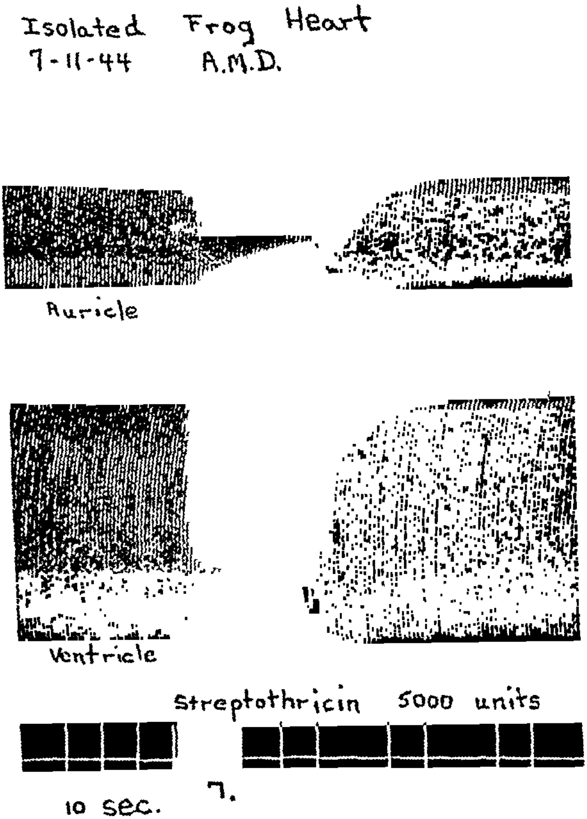

Fig. 5. Influence of Streptothricin (Potency 200 Units Per mgm.) on the Isolated Frog Heart

Streptothricin removed from cannula and replaced with Ringers solution after 20 seconds.

Discussion. From the foregoing data it is apparent that in its present form form streptothricin produces a number of toxic side effects following oral and parenteral administration to animals. The nature of these toxic signs would seem to preclude its use for the treatment of systemic infections in man.

The toxic signs manifested by animals injected with streptothricin were characterized by acute cardiovascular disturbances which were similar in many onset of congestion and edema, followed 24 to 48 hours later by suppuration. This reaction was still evident one to two weeks after applying the drug to the eye; the palprebral fissure being sealed shut with a mucoid mass of pus. Examination of the cornea following the application of flourescein revealed marked destruction of the cornea.

Intradermal injection in guinea pigs. Here again neutral solutions of *streptothricin F or W* were found to produce irritation when concentrations of 500 units per cc. were injected into the shaved abdomen of guinea pigs. Greater concentrations led to severe local irritation which progressed to scar formation.

Body temperature. Therapeutic doses of *streptothricin F or W* had no influence on the body temperature of mice, rats, rabbits or dogs when administered by intravenous injection. Larger doses (10,000 units per kgm.) of certain batches caused a typical thermal response in rabbits and dogs indicating the presence of pyrogens. Lethal doses produced a fall in body temperature by several degrees.

Liver function. Liver function tests performed in dogs by the bromsulfalein method of Rosenthal (17) showed that large doses of streptothricin had no apparent effect on the liver even in animals approaching death providing that the normal functioning of the cardiovascular system was maintained. Direct and indirect van den Bergh tests were normal indicating the normal excretion of bile pigments.

Effects on isolated organs. Intestine. Both forms of streptothricin caused a marked relaxation of the smooth muscle fibers of the rabbit intestine. Moreover, *streptothricin F and W* were capable of promptly counteracting the spasm produced by barium chloride. When the intestinal strip was washed with Locke's solution, normal peristalsis was resumed. These findings are opposite to those produced by histamine.

Isolated frog heart. Streptothricin W, in concentrations of 200 units per cc. of Ringer's solution caused the heart to stop in diastole. This effect was not overcome by increasing the hydrogen ion concentration and calcium of the Ringer's solution. Lower concentrations also reduced the amplitude of the heart beat although eventually the heart recovered (figure 5).

Isolated rabbit ear. The instillation of small amounts of *streptothricin F* (500 units) into the vascular system of the isolated rabbit ear generally caused a significant relaxation of the blood vessels. This effect, however, was readily *overcome by the injection of dilute solutions of adrenalin (1:50,000), occasionally*, however, constriction of the vessels was obtained. These findings are in agreement with the results *in situ* in which it was found that *streptothricin F* generally caused a marked dilation of the peripheral vascular system with occasional variable results in rabbits.

Absorption and excretion. The findings presented in figures 6 and 7 show that *streptothricin W and F* are rapidly absorbed and excreted by the animal body. Most of the drug could be accounted for in the urine within 5 to 6 hours after drug administration. However, therapeutically effective blood concentrations of the drug can be maintained by repeated administration at frequent respects to those caused by histamine or by low molecular weight peptones and albumoses (16). However, it was demonstrated that histamine was not present in streptothricin preparations as evidenced by the resistance of the depressor substance to histaminase.

It is of interest to note that the *streptothricin F* obtained from the medium designed by Foster (6) contained much greater amounts of this depressor substance than that obtained from the medium of Waksman (5). Since there was no relationship between the active bacteriostatic principle and the depressor substance, the latter appears to be a separate metabolic product liberated by *Actinomyces levenulae*. It would seem therefore that the two substances might be separated by appropriate chemical means thus eliminating one of the toxic products of streptothricin.

More serious than the foregoing toxic effects were those manifested by animals five to twelve days after the injection of lethal doses of streptothricin. Death in these cases appeared to be due to renal insufficiency as evidenced by the complete suppression of urine excretion 24 to 48 hours before death. According to Mushett and Martland (18), dogs, monkeys and rats injected with lethal doses of streptothricin showed degenerative changes in the kidneys which varied from cloudy swelling and fatty degeneration to extensive tubular necrosis. Changes in the glomeruli were relatively infrequent. It would appear therefore that streptothricin in its course of being excreted by the kidneys induces certain toxic effects which eventually lead to the foregoing pathological changes and renal insufficiency. Concurrent with these renal changes, one observes a rise in the N.P.N. and blood urea concentration thus affording further evidence of renal impairment. It is of interest to note that the onset of these reactions is delayed much in the same manner as are the local irritating effects caused by applying streptothricin to the rabbit eye. Since streptothricin has been shown to be excreted from the animal body within the first three to five hours after parenteral administration, the toxic effects would appear to be induced during this time. These first effects apparently do not impair renal function as evidenced by blood chemistry, diuresis experiments and the normal behavior of the animal. However, during the course of the following five to twelve days renal function gradually becomes more impaired and ceases just before death. Studies to date indicate that this toxic factor may be associated with the active bacteriostatic principle since impure (50 units per mgm.) and relatively pure (500 units per mgm.) samples caused the same toxic signs. However, it is also possible that the present chemical methods employed for purifying streptothricin are not adequate for separating the nephrotoxic factor from the bacteriostatic principle. Therefore a final statement in this matter can not be made until the pure drug is available for study.

CONCLUSIONS

1. Crude streptothricin was found to be relatively toxic for mice, rats, guinea pigs, rabbits, dogs and monkeys. Doses approximately 5–10 times the therapeutic dose were lethal.

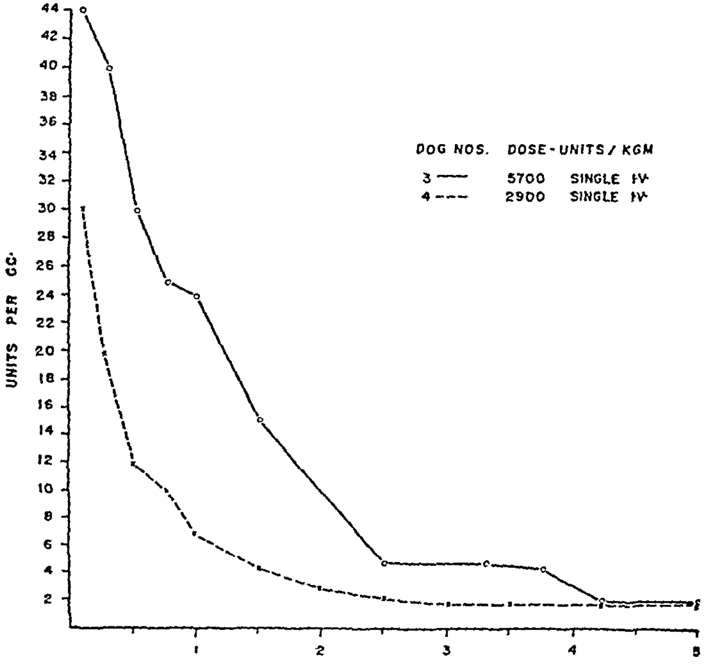

FIG. 6. STREPTOTHRICIN LEVELS IN THE BLOOD OF THE DOG

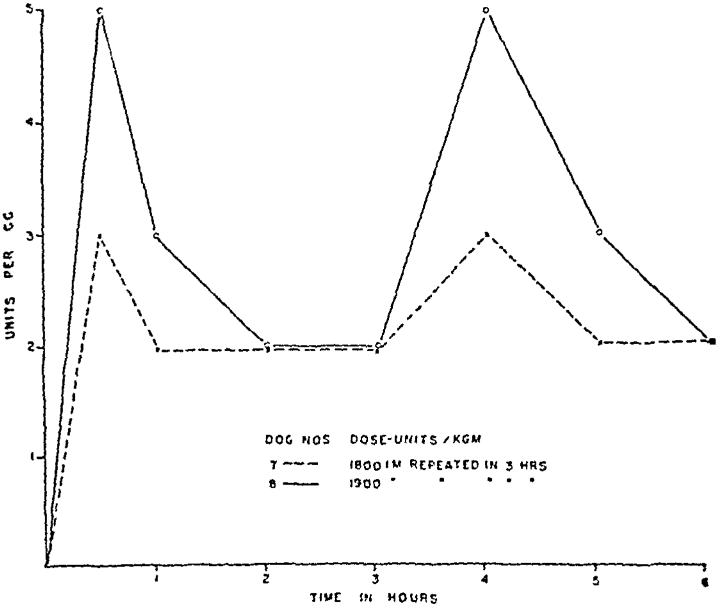

FIG. 7. STREPTOTHRICIN LEVELS IN THE BLOOD OF THE DOG

THE CHRONIC TOXICITY OF SULFITES

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For many years the process of sulfurizing has been the principal commercial method of preserving dried fruits and for an equal number of years the question of the harmful potentialities of sulfites in food products has lacked a definite answer based on scientific experimentation and observation. The work of Wiley (1) on human volunteers, which was performed toward the beginning of the present century, indicated but produced no conclusive evidence that metabolic disturbances resulted from the addition of salts of sulfurous acid to foods. The older experiments of Kionka (2) and of Lehman (3) showed that large, although unspecified amounts of sulfites, added to meat diets of dogs produced diarrhea and kidney damage. Rost and Franz (4) failed to confirm the results of Kionka. They gave 40 dogs, both growing and mature, oral doses of different sulfites varying from 0.05 to 1 gram per day for periods of from 1 month to over a year without visible macroscopic or microscopic tissue changes. Larger doses caused vomiting; however, no other symptoms appeared. In humans they found that doses of 1 gram of sodium sulfite per day decreased the utilization of protein and fat. Gastrointestinal symptoms seldom occurred at this dose, but with doses of from 4 to 5.8 grams per day there occurred abdominal pains and vomiting.

Later experiments of Morgan and her associates (5, 6) showed the ability of sulfites to destroy thiamine. This fact has been of such importance that in any calculation of the daily allowance of this vitamin the theoretical amount in dried fruits has had to be totally disregarded. Recently, in order to supply the special food requirements of troops fighting in very hot climates, it was necessary to sulfurize fruits to an extent where any toxic hazard inherent in the process, in addition to the destruction of thiamine, became an important problem. The experimental work reported below was conducted for the purpose of evaluating the toxicity of various levels of sulfites when added to the diet. Since sulfites may be converted to sulfides and sulfates these were included in our studies as well as diets containing sulfite but low in thiamine and diets with sulfites and added thiamine.

Experimental. Rats from our colony of Osborne-Mendel strain have been used in 3 experiments on the chronic toxicity of sulfites. The ration used in each experiment was composed of corn starch 60%, casein 18%, corn oil 6%, brewer's yeast 5%, whole liver powder 5%, salt mixture (U.S.P. XII No. 2) 4%, and cod liver oil 2%. All diets were refrigerated until used. The rats were kept in individual cages in a room with the temperature and humidity controlled.

The first experiment hereinafter referred to as the 1940 experiment was designed to study the effect of thiamine on the toxicity of sulfites. The supply of thiamine for the rats was either increased above or decreased below that found in the regular diet. The increase in available thiamine was by intramuscular injection of 100 micrograms twice

2. The pharmacodynamic action of streptothricin appeared to be due to the presence of two toxic factors. The first caused marked acute changes in the cardiovascular and respiratory system of rats, rabbits, cats and dogs and in this respect appeared to be similar to histamine. This active principle, however, does not appear to be histamine. The second toxic factor appeared to be related to the active principle and induced certain pathological changes in the kidney which led to renal insufficiency and death a number of days after the drug was excreted from the body.

3. Aqueous solutions of streptothricin containing 200 units per cc. or more caused marked inflammation when applied to the rabbit eye over a 30 minute interval. This concentration is far above the bactericidal concentration.

4. Streptothricin is absorbed and excreted rapidly following parenteral administration to mice, rats, rabbits, dogs and monkeys.

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Such comparisons are made in this study. For the 52-week period, the design of the experiment was upset by the death of a large number of animals and conclusions are based only on those rats that survived. Since no deleterious effects on growth were caused by the sulfates or sulfides, analysis will be shown on the sulfites only.

The comparisons are presented graphically. Figure 1 shows the average weights over 12 weeks according to diet, as labeled; males and females are given separately since they have decidedly different averages and standard deviations. Limits are shown bracketing the average weights. The limits are to be used in comparing the average weight for each experimental diet with the average weight for the control group, and show the size of difference from the control that would be statistically significant (p = 0.05) in this experiment. Where the numbers of experimental and control animals are the same, as is the case of most of the 12 week data, the brackets can also be used for judging the significance of the difference between two test diets. The limits are calculated using the

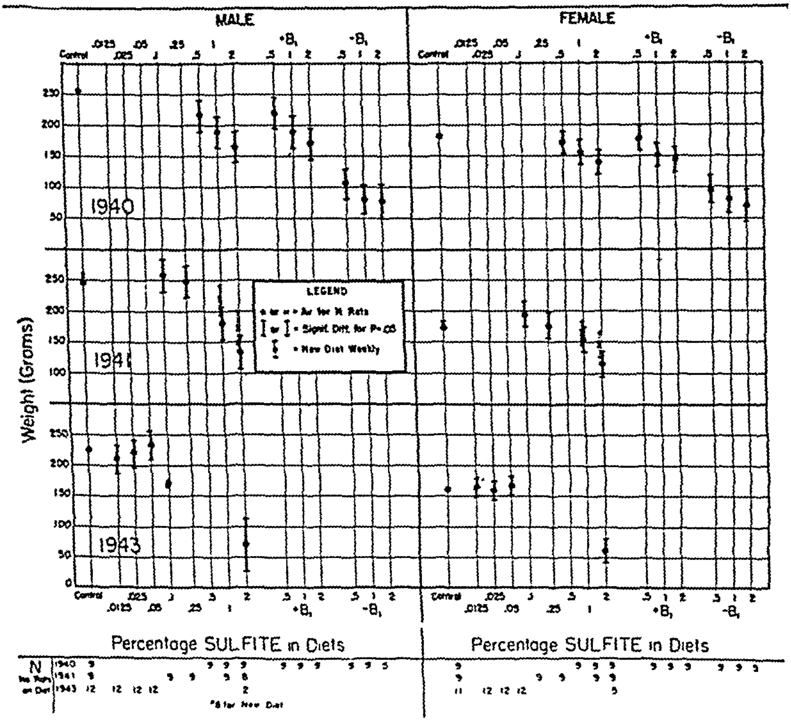

FIG. 1. AVERAGE WEIGHTS OF RATS OVER THE PERIOD OF THE FIRST 12 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS

weekly. The available thiamine was decreased by autoclaving the yeast before adding it to the diet. The sulfite was added as sodium bisulfite and the sulfite values referred to throughout are to this salt ($NaHSO_3$) and not to sulfur dioxide. In this experiment 3 levels were used: 0.5%, 1% and 2%, in 3 different types of diets. The first diet had sulfite added, the second contained sulfite and was supplemented with thiamine, and the third contained sulfite but had its thiamine content reduced by autoclaving the yeast component. A fourth untreated diet was used as a control thus making a total of 10 diets. The experiment utilized a balanced incomplete block design where each of 3 diets was fed to each of 3 rats from the same litter. The design was replicated for male and female rats. Thus 3 male and 3 female rats were taken from each of 30 litters at 3 weeks of age and placed at random on the proper diet according to the design.

Almost a year later when it appeared that aging of the diets containing sulfites was having some effect, a second experiment hereinafter referred to as the 1941 experiment was begun to compare the effects of the various levels of sulfite in diets prepared weekly with the effects of the same levels in diets prepared in sufficient quantities to last five or six weeks and stored under refrigeration. These diets, designated "old" and "new", were used for the controls and the 1% and 2% sulfite levels. Two lower levels of sulfite, namely 0.1% and 0.25%, were also included on the "old" diet basis. Since sulfite is sometimes oxidized to a sulfate, two levels of sodium sulfate (0.25% and 1%) were included in this experiment. Thus again, 10 diets were used in the same type of balanced incomplete block design as utilized in the 1940 experiment.

A third experiment hereinafter referred to as the 1943 experiment was begun in which lower levels of sulfite were included (0.0125%, 0.025%, 0.05% and 2%). Since diarrhea with black feces resulted from feeding sulfites at the high levels it was considered possible that a sulfide might be contributing to the toxicity of the sulfite; therefore, two levels of sodium sulfide (0.25% and 1%) were fed. Seven diets including the control were used in this experiment. The proper replicated balanced incomplete block design utilized 28 litters with 3 males and 3 females from each litter and resulted in 12 males and 12 females on each diet.

RESULTS. *The effect on growth.* Usually comparisons of effects of various diets on growth over a period of time are made by comparing the gains in weight. However, this results in a loss of much information on the growth curve of the rat as given by the weekly weighings. In the following analyses an increase in information on the growth effects has been obtained by including both the gain in weight and the average weight for the individual animal over the period of study. Since the rats from each litter on this experiment are assigned to the diets at random and since the initial weights are within rather small limits, no correction for initial weight is necessary.

Analysis of the growth curves for this study was divided into 2 parts: (1) the first 12 weeks and (2) the first year. For the first 12 weeks, mathematical curves were initially fitted to the weekly weights of each rat. These curves were of such a type (orthogonal polynomials) that the several coefficients for one curve were independent of each other (7). The first coefficient is the average weight of the rat over the period; the second coefficient is proportional to the average gain in weight; the third coefficient is proportional to the average change in gain in weight, etc. In this study, the first two coefficients were the only ones that showed consistent trends with increased doses of sulfites. This, therefore, is equivalent to analyzing the effects of the various levels of sulfites in the diet by comparing average weights, and by comparing gains in weight.

in all instances for the 0.0125% of sulfite) this can be interpreted to mean that in this experiment there is no significant difference between the average weights for the control and that level of sulfite. If the average for the control falls outside the limits a statistically significant difference exists.

The charting of results in this manner points out not only significant differences from the control for all diets simultaneously but also any trends of average weight or gain in weight with increasing dosages of sulfite. Moreover, visual comparisons can be made of results for several experiments.

Figures 1 to 4 show the following about the average weights and the average gains in weight of the rats on these three experiments:

1. There is a very definite trend toward smaller average weights and smaller gains in weight with an increase of sulfite in the diet above 0.25%
2. The rats on the diets containing 0.05% and 0.1% sulfite grew more rapidly and had greater gains in weights for the first 12 weeks than the rats on the control diet. The average weights and gains in weight shown on figures 1 and 2 and the average weights shown on figure 3 are slightly higher for the animals on the above levels than the control for both male and female rats. Considering the 12 week data first there are 8 comparisons (figures 1 and 2) between the control group and the experimental groups fed these two levels of sulfite and as can be seen from the figures all differences are in the same direction. These early gains were not maintained up to the end of the first year. Thus at 52 weeks the male rats on the 0.1% sulfite-containing diet had a gain in weight significantly smaller than the control as can be seen on figure 4. The female rats also had smaller gains in weight for the 52 week period though the difference is not significant. Rats on the 0.05% sulfite level had gains in weight which did not differ significantly from the controls at 52 weeks.
3. The results on the series having supplemental injections of thiamine were about the same as on those without the added thiamine.
4. The rats on diets from which the greater part of the thiamine had been removed had the lowest series of average weights and gains in weight. However, there is also a downward trend with increased amounts of sulfite in this diet.
5. For the 12 week period rats on the "new" diet had larger average weights and gains in weight than those on the "old" diet. Although these differences are not individually significant, they were all in the same direction.

The effect on survival time. The increased amounts of sulfite in the diet affect not only the growth of the rats on these diets but also the survival time. Since

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number of animals in all the groups used to obtain the pooled variances σ^2.) The limit $\bar{x}_e - \bar{x}_i$ for $p = 0.05$ used on figures 1, 2, 3, and 4 is found by transposing the above formula to

$$\bar{x}_e - \bar{x}_i = t\sigma\sqrt{\frac{1}{N_e} + \frac{1}{N_i}}$$

and the value of t is obtained from a "t" table for $p = 0.05$ and degrees of freedom equal to $N_e + N_i - 2$. A "t" table is given in almost any statistical text or book of statistical tables (8).

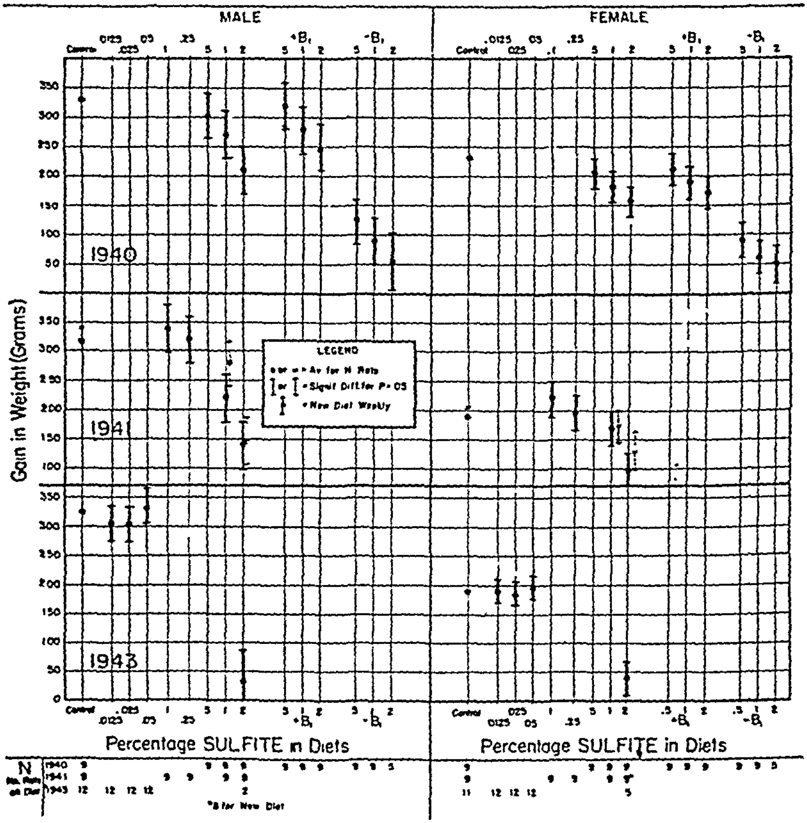


FIG. 2. GAINS IN WEIGHT OF RATS OVER THE PERIOD OF THE FIRST 12 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS

formula for the "t" test.[1] If the average for the controls falls within the limits designated on either side of the average for a particular level of sulfite, (as it does

[1] In application of the formula for the "t" test,

$$t = \frac{\bar{x}_c - \bar{x}_i}{\sqrt{\frac{\sigma_c^2}{N_c} + \frac{\sigma_i^2}{N_i}}}$$

$\bar{x}_c$ and $\bar{x}_i$ are the average weights for N_c and N_i animals on the control and experimental diet "i" respectively. Also σ_c^2 and σ_i^2 are equal and are the pooled variances (squares of the standard deviations) for all groups where there is no correlation between the average and the variance. (The square of the standard deviation is calculated for the average weights on each experimental and the control diets. These variances are then each multiplied by the number of animals N in each group and sum of these products is divided by the total the addition of sulfates and sulfides has no effect on the survival time of the animals, analyses are shown only on the sulfites. Figure 5 shows the diets in the same order as in figures 1 to 4, with the results for male and female rats shown separately. In figure 5 each symbol represents one rat and the averages of results on each diet are indicated by horizontal lines. No significant limits are indicated since the rats that did survive were killed at the end of that experiment as shown by the bunching of symbols at the top of the 1940 and the 1943 parts of the figure and since the distribution of survival times followed none of the commonly recognized types. Each of the 3 experiments was continued for a different length of time, the 1940 experiment for one year, the 1941 experiment for one and one-half years, and the 1943 experiment for 2 years. However, the trends shown are very similar to those shown in figures 1 to 4. In all cases the rats on a diet containing 2% of sulfite had a short average survival time. In

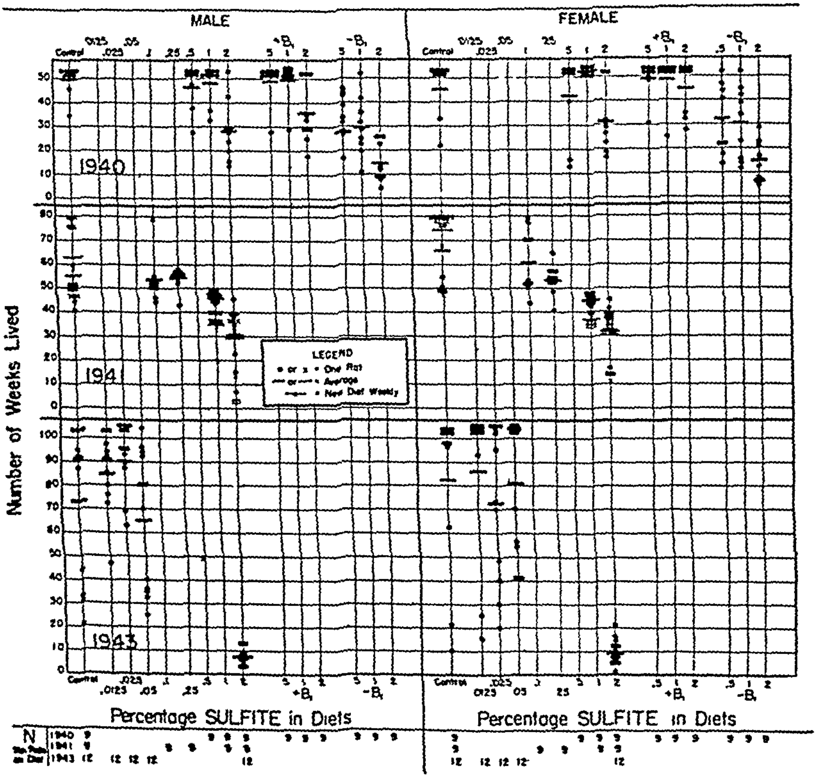


FIG. 5. SURVIVAL TIME FOR RATS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES —3 EXPERIMENTS

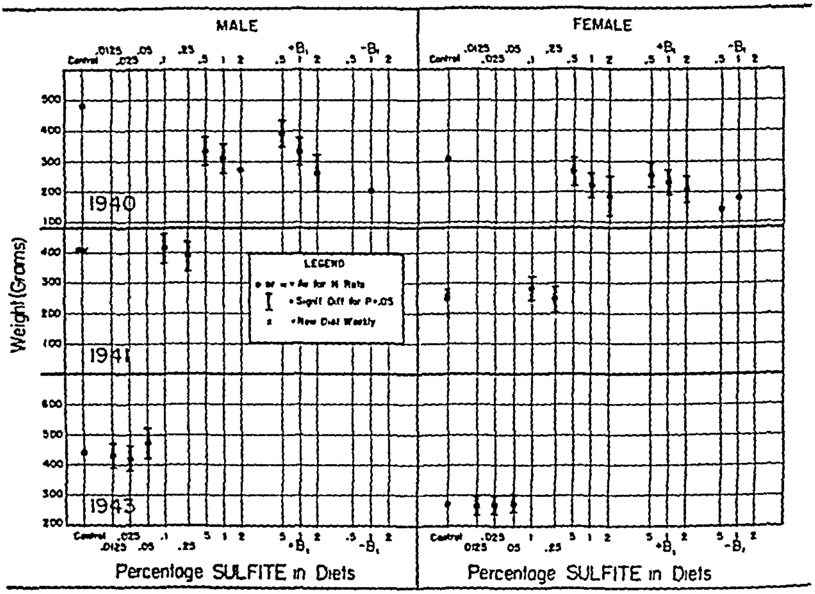
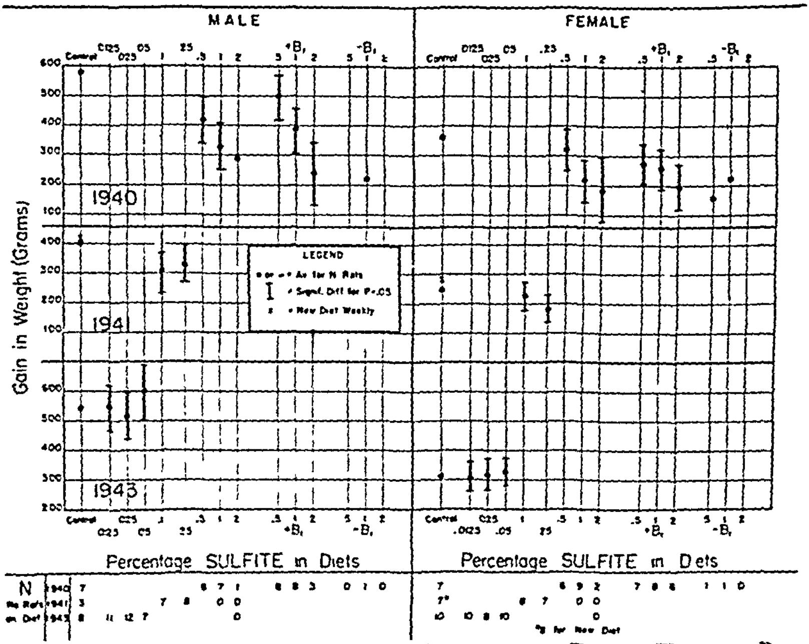


FIG. 3. AVERAGE WEIGHTS OF RATS OVER THE PERIOD OF THE FIRST 52 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS



IG. 4. GAINS IN WEIGHT OF RATS OVER THE PERIOD OF THE FIRST 52 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS

TABLE 1

Summary of gross and microscopic pathology in rats fed sulfite, sulfate or sulfide

DOSAGE*	ANIMALS IN GROUP	POLYNEURITIS	BLEACHED TEETH	SPECTACLE EYES	BROWN UTERI	HAIR BALLS IN STOMACH	GASTRIC EPITHELIAL HYPERPLASIA	MYOCARDIAL FIBROSIS	FATTY DEGEN OF LIVER	CALCIFIED RENAL TUBULAR CASTS	RENAL EPITHELIAL PIGMENT	TUBULAR ATROPHY OF TESTIS
1940 series												
2% − B_1	18	16	0	4	0/18	2/18†	4/18	3/15	0/15	11/15	0/15	Mod. to marked
2%	18	2	3	9	0/18		1/18	4/12	2/12	10/12	2/12	Mod. to marked
2% + B_1	18	0	8	9	1/18		0/18	3/12	0/12	9/12	6/12	Mod to marked
1% − B_1	18	17	0	0	0/18		0/18	4/12	0/12	10/10	2/10	Mod. to marked
1%	18	0	1	1	3/17		0/17	1/10	0/10	7/10	9/10	Mod. to marked
1% + B_1	18	0	0	0	0/18		0/18	0/10	0/10	7/10	8/10	Mod. to marked
0.5% − B_1	18	16	0	0	0/17		0/17	3/11	1/11	10/11	0/11	Mod. to marked
0.5%	18	0	0	1	1/17		0/17	3/10	1/10	5/10	3/10	Mod. to marked
0.5% + B_1	18	0	0	0	1/18		0/18	2/11	2/11	5/11	6/11	Mod. to marked
Control	18	0	0	0	0/18		0/18	1/13	4/13	1/13	1/13	Slight
1941 series												
2%, "old" diet	18	7	7	2	4/17	†	6/17	3/11	1/11	9/11	2/11	Mod. to marked
2%, "new" diet	18	9	0	1	0/17		2/17	2/10	0/10	9/10	0/10	Mod. to marked
1%, "old" diet	18	5	0	1	7/18		1/18	1/9	0/9	1/9	1/9	Mod. to marked
1%, "new" diet	18	8	0	2	1/17		1/17	0/9	1/9	8/9	0/9	Mod. to marked
0.25% "old" diet	18	6	0	0	1/17		6/17	0/9	3/9	5/9	1/9	Mod. to marked
0.1% "old" diet	18	3	0	1	0/18		7/18	0/9	2/9	3/9	0/9	Mod. to marked
Sulfate 1%	18	0	0	0	0/15		0/15	2/7	5/7	4/7	0/7	Moderate
Sulfate 0.25%	18	0	0	0	0/17		0/17	0/6	4/6	1/6	0/6	Slight
Control, "old" diet	18	0	0	0	0/9		1/9	1/7	5/7	2/6	1/6	Slight to moderate
Control, "new" diet	18	0	0	0	0/9		1/9	0/6	3/6	2/6	1/6	Slight to moderate
1943 Series												
0.05%	24	0	0	0	0/22	‡	0/22	2/16	10/16	3/16	0/16	Slight
0.025%	24	0	0	0	0/17		0/17	3/8	3/8	0/8	0/8	Slight
0.0125%	24	0	0	0	0/19		0/19	0/9	6/9	1/9	1/9	Slight
Sulfide 1%	24	0	0	0	0/15		1/15	0/10	5/10	2/10	0/10	Slight
Sulfide 0.25%	24	0	0	0	0/17		1/17	1/9	6/9	1/9	0/9	Slight
Control	24	0	0	0	0/13		0/13	2/8	5/8	0/8	0/8	Slight

* Sulfite unless otherwise specified.

† In 1940 and 1941 series present in from ⅓ to all of autopsied rats in all groups except 2% − B_1. More frequent in second series than in first.

‡ Present in from ⅓ to ½ of autopsied rats in all groups of 1943 series.

1941 even the 0.25% sulfite in the diet seems to have shortened the lives of the female rats and the 0.1% sulfite in the diet also produced an average survival time less than the control No trends are evident for survival times of rats on diets containing less than 0.1% sulfite. The results on diets supplemented by thiamine seem to be about the same as those not supplemented, whereas the reduction of the thiamine in the diet resulted in a sharply decreased average survival time.

Pathology. Of the 504 rats started on these experiments, 70 were not sent to the pathology laboratory for one reason or another. Of the remaining 434, 258 were sectioned microscopically. Sections were made routinely of lung, heart, liver, spleen, pancreas, stomach, small intestine, kidney, adrenal and testis, with other structures such as ovary, uterus, colon, brain, thyroid, bone, bone marrow and leg muscles being sectioned less frequently. In addition, certain other animals had confirmatory sections made of spontaneous tumors and other noticeable conditions, but these are not considered as sectioned animals.

Rats fed high doses of sulfite in the diet frequently showed stunting of growth, clinical polyneuritis, "spectacle" eyes, bleached incisor teeth, brown uteri, atrophy of various viscera, calcified renal tubular casts, and atrophy of bone marrow and bones; less frequently they showed focal myocardial necrosis and fibrosis, and gastric squamous epithelial hyperplasia (table 1). With a decrease in the percentage of sulfite from 2% to 0.5% or even to 0.25% there was not a great deal of change in the lesions observed; the animals simply lived longer and finally attained about the same condition that those on higher dosage levels did in a shorter period. This is, however, a broad general statement and will not be exact for each individual lesion. The level of sulfite in the diet which would begin to cause histological changes in rats would appear to lie at approximately 0.1% on the basis of these studies.

Aging of the diet had a definite effect on incidence of lesions of the teeth and uteri, while it had no effect on the polyneuritis; this indicates a sharp separation in etiology.

The following remarks are intended to help clarify the nature and degree of involvement of some of the lesions listed in table 1. Hyperplasia of the gastric squamous epithelium (usually near its junction with the glandular portion of the stomach) somewhat paralleled the browning of the uteri, although not closely. The lesion was slight or moderate in degree in all instances. "Myocardial fibrosis" included focal lesions in all stages from recent necrosis to replacement by fibrous tissue. The left ventricle was chiefly affected, the right ventricle less so, and the atria scarcely at all. The lesion was slight or moderate in degree in all instances. Fatty degeneration of the liver was very slight or slight in all instances except a few of moderate degree. It will be noted that the condition was more frequent in the control, sulfate, sulfide, and low dose sulfite rats than in rats receiving high doses of sulfite. There was no apparent relation between thiamine and this condition in the present material. Calcified renal tubular casts occurred chiefly around the corticomedullary junction. Very little tubular atrophy occurred as a result of the process. Brown pigment in the renal convoluted diarrhea may have prevented the synthesis or absorption of the biotin in the intestinal tract.

Chemical analyses of the diets showed that up to 75% of the sulfite disappeared when the diets were left open to the air at room temperature for a week. A part of this was accounted for by an increase in sulfate sulfur in the diet and the other part probably escaped as sulfur dioxide. Less than 20% was lost from diets refrigerated for as much as 6 weeks. Since the diets were exposed in the food cups to the air for varying lengths of time up to a week, these facts indicate that the animals obtained only part of the sulfite originally added to the diet.

It is observed from the experiment with the added thiamine that a depletion of the antineuritic vitamin is not the only factor affecting growth rate. These rats had no polyneuritis; however, they showed a distinct growth retardation. A deficiency either of vitamin E or of biotin, or a combination of the two, may have produced this effect. With the possible exception of a reduction in the amount of biotin made available to the animal as a result of feeding sulfite, our results support the finding of Kline (16) who stated that only thiamine of the vitamin B complex was destroyed during the process of treating dried yeast with a solution of sodium sulfite.

Testicular atrophy, renal tubular calcified casts and gastric squamous epithelial hyperplasia had a complex etiology in this study, with inanition, vitamin deficiency and perhaps direct sulfite toxicity all being partially responsible for the conditions observed. Hair balls in the stomach were unusually frequent in these animals, even in the controls. They are rarely seen in control animals of other experiments and indicate some induced deficiency in all diets, as do the mild fatty degeneration of the liver and the testicular atrophy. The diarrhea observed in all rats on concentrations of sulfite at 0.1% or more was specific for the sulfite, and resulted possibly from an irritation of the gastrointestinal tract. It is difficult to draw a line of demarcation between the effects due to ingestion of the sulfite residues and those due to the consumption of a diet rendered deficient by the addition of sulfite. Contrary to our expectations neither sulfate nor the sulfide showed any laxative action.

SUMMARY

1. Sulfite in concentrations of 0.1% (615 p.p.m. as SO_2), or more, in the diet was toxic to rats. Amounts of sulfite less than 0.1% (615 p.p.m. as SO_2) in the diet had no significant effect on growth. The rats on the 0.05% (308 p.p.m. as SO_2) sulfite containing diet grew more rapidly and had greater gains in weight for the first 12 weeks; however, these gains were not maintained up to the end of the year.

2. There was a definite trend toward smaller average weights and smaller gains in weight of the animals as the sulfite in the diet increased from 0.1% to 2%.

3. Rats fed diets containing sulfite with the addition of thiamine given hypodermically showed growth curves very similar to those of rats on these same diets without the added thiamine.

tubules, most of which did not react for iron with acid ferrocyanide, was most frequently seen in the groups receiving added thiamine. The condition was always of slight or moderate degree. Testicular tubular atrophy of itself showed no special features. Intertubular edema was seen almost entirely in the first series and principally in the sulfite and sulfite plus thiamine groups. On a dosage level of 2% the majority of the testes in these two groups showed from slight to marked edema.

Lung, adrenal, pancreas, small intestine, colon, ovary, and spleen showed no distinct differences between test and control animals attributable to the sulfite, the only changes being those of inanition. A few small ulcers in the proventriculus were scattered among the various groups; in all except one of these stomachs hair balls were present. A few each of brain, sciatic nerve and thyroid were sectioned. These were negative except for one brain showing hemorrhages and one with foci of softening, in animals with polyneuritis. Tibia and femur, including the bone marrow and the surrounding leg muscles, were sectioned more frequently; the only changes observed here were those of inanition.

The animals receiving sulfates and sulfides in their diets showed no distinct differences from the controls (table 1) as a result of the treatment.

DISCUSSION. The greater share of the deleterious effect of sulfites in the diet is probably due to the destruction of vitamins. In studies on the chemistry of thiamine, Williams, Waterman, Keresztesy and Buchman (9) reported that sulfite split the molecule and destroyed the antineuritic properties of this vitamin. This observation would explain growth changes and much of the pathology observed in the present experiment. The destruction of the thiamine in the diet caused the polyneuritis and most, if not all, of the focal myocardial necrosis and fibrosis. Since a lack of thiamine decreases the desire for food (10, 11), the inanition which produced the stunting of growth and atrophy of the individual organs and tissues can be considered to be a result of destruction of the vitamin by the sulfite. Depigmentation of the teeth, brown uteri and "spectacle" eyes have not been reported to be caused by a thiamine deficiency. Moore (12) stated that either a vitamin E or vitamin A deficiency will cause dental depigmentation in the rat. The fact that aging of the diets had no greater effect on growth indicates that the diets were not low in vitamin A. In this connection Morgan (5) reported that the process of sulfurizing dried fruits did not destroy vitamin A. Further studies are being made on the effects of sulfite on the teeth and the findings will be reported later. According to Mason (13) female rats with a vitamin E deficiency of long duration have brown uteri and the intensity of the color increases as the deficiency continues. Although the brown uteri in the present experiment were grossly and microscopically of the type generally considered typical of vitamin E deficiency, they did not have the intense color which we have observed in rats with definite vitamin E deficiency (14). No other uncomplicated histopathological evidence of vitamin E deficiency was seen. "Spectacle" eyes in rats have been reported to occur in biotin deficiency (15). Since the rats on the levels of sulfite which caused this symptom also had extreme diarrhea, either the diarrhea or the condition in the intestines that produced the

THE ACTION OF PICROTOXIN AND OF NIKETHAMIDE (DIETHYLAMIDE OF PYRIDINE-3-CARBOXYLIC ACID) ON NEUROMUSCULAR CONDUCTIVITY

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Of the drugs called "stimulants of the central nervous system", the only ones which have been studied in the peripheral nervous system, from the viewpoint of the chemical theory of the transmission of the nerve impulse, are strychnine, metrazol, and caffeine. Lanari and Luco (1939) state that strychnine produces a depression of neuro-hormonal transmission, both in skeletal muscle and in the superior cervical ganglion. Croxatto and Alonso (1940) and Salvestrini, Croxatto and Alonso (1940) have shown that metrazol depresses the synapses of the superior cervical ganglion and the neuromuscular junctions. These authors call attention to the pharmacological similarity between strychnine and metrazol as regards phenomena of neural conductivity. On the other hand a study made in this department (Huidobro and Amenabar, 1945) showed that caffeine enhances the transmission of the nerve impulse both in the superior cervical ganglion and in the neuromuscular junction. Strychnine and metrazol inhibit the passage of the nerve impulse by increasing the threshold of response to acetylcholine; on the contrary, caffeine facilitates the conduction of nerve impulses by its lowering effect on this threshold.

We have thought it to be of interest to study the effect of picrotoxin and nikethamide on the neuromuscular junction since these two substances are also also central nervous system stimulants. Picrotoxin, moreover, is a strong convulsant, as are strychnine and metrazol. On the other hand, nikethamide, when given in toxic doses, has more of an analeptic action such as that of caffeine. (See Goodman and Gilman, (1941)).

METHODS. The animals used were cats anesthetized with sodium pentobarbital (Nembutal, Abbott: 0.033 Gm. dissolved in 1 cc. of 25% urethane, per kilogram body weight, intraperitoneally). A tracheal cannula was inserted so that artificial respiration might be given if necessary. In the studies on nikethamide the quadriceps femoris and soleus were used interchangeably, whereas in the case of picrotoxin the quadriceps was used exclusively. Both muscles were stimulated indirectly, the respective nerves being isolated and divided or clamped proximally to the point of stimulation. Silver electrodes insulated with rubber were used. Condensers controlled by vacuum tubes were employed as the source of stimulating charges. The stimuli applied were in all instances maximal. Muscular contractions were registered mechanically on smoked drums. For this purpose the femur of tibia (depending on whether the quadriceps or soleus was used) was fixed with clamps and the distal tendon of the muscle, having been isolated and divided, was fastened to the short end of a lever which exerted traction on elastic bands.

In some instances the muscle was stimulated directly. In such a case it was denervated 4 to 7 days previously. Stimulation was obtained from a Harvard induction coil with 10 volts in the primary circuit, the distance between the primary and secondary being deter-

4. The addition of sulfite to the diets produced deleterious effects over and above those produced by the removal of the main source of thiamine.

5. Sulfite in concentrations of 0.25% (1538 p.p.m. as SO_2), or more, decreased the average survival time.

6. The lowest level of sulfite in the diet which produced histopathological changes was 0.1% (615 p.p.m. as SO_2).

7. The gross and microscopic pathological changes in the rats on 0.25% (1538 p.p.m. as SO_2) or more, of sulfite in the diet included stunting of growth, clinical polyneuritis, "spectacle" eyes, bleached incisor teeth, brown uteri, atrophy of various viscera, calcified renal tubular casts, atrophy of bone marrow and bone, and less frequently focal myocardial necrosis and fibrosis and gastric squamous epithelial hyperplasia.

8. Aging of the sulfite-containing diets caused a decrease in growth rate during the first 12 weeks and an increase in the incidence of lesions of the teeth and uteri, while it had no significant effect on the incidence of polyneuritis.

9. Sulfate and sulfide in the concentrations used in this experiment produced no growth or histopathological changes.

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With a higher frequency of stimulation, about 10 to 20 per second, nikethamide has a slight tendency toward the production of depression in muscular contractions. Only from time to time is there seen any increase in muscular tension.

If the muscle be stimulated with such high frequencies as 20 to 100 per second, the effect of nikethamide on the indirectly stimulated muscle is always one of decided depression. Recovery of the muscle tension to its original level is complete by the end of one or two minutes, or occasionally, a little longer (figs. 1 and 2). Only rarely is there a failure of recovery.

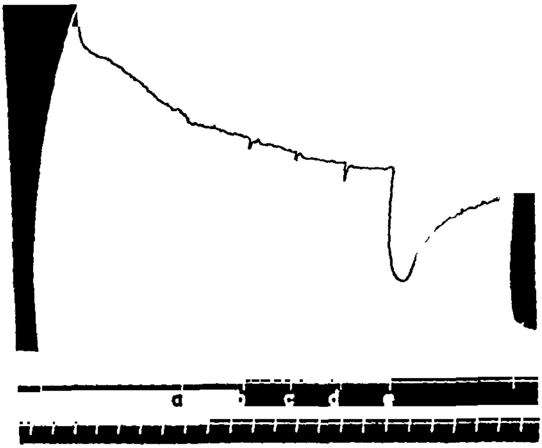


FIG. 1. ACTION OF PICROTOXIN AND NIKETHAMIDE ON INDIRECTLY STIMULATED MUSCLE

A cat anesthetized with a strong dose of nembutal. Maximal stimulation of the quadriceps femoris muscle through the crural nerve with a frequency of 32 per second. Upper tracing: muscle. Upper line: first and last signal, onset and end of stimulation; a) intra-aortic injection of 0.1 mg. of picrotoxin; b) intra-aortic injection of 0.2 mg. of picrotoxin; c) intra-aortic injection of 0.35 mg. of picrotoxin; d) intra-aortic injection of 0.5 mg. of picrotoxin; e) intra-aortic injection of 50 mg. of nikethamide. Lower line: time in minutes.

When a muscle is indirectly stimulated at different frequencies, the graph of muscular contraction shows a series of deflections which have been designated stages of neuromuscular transmission. Various stages have been described; namely, first, second, 3a, 3b, 3c, fourth and fifth. (For a description of this phenomenon and references, see Rosenblueth and Cannon, 1940.) In our studies thus far described the nikethamide has been injected in the 3c stage or in the early part of the fourth, or in a state which was either 3c or fourth, but could not be definitely classified, as there is some confusion between the stages when one uses a fixed frequency of stimulation. We thought it of interest to observe the mined so as to deliver maximal stimuli. Steel needles were used as electrodes, one being placed in the tendon and the other in the muscle belly. In other experiments the muscle was stimulated with acetylcholine. This drug was injected into the abdominal aorta after ligation of the contralateral iliac artery, the medial sacral artery, and the inferior mesenteric artery, in animals in which the muscles had been aspetically denervated eight to ten days previously.

The drugs used were: Picrotoxin (Eimer and Amend) in 2% solution; nikethamide (Coramine, Ciba; the diethylamide of pyridine-3-carboxylic acid) in 25% solution; atropine sulfate (Merck) in 10% solution; acetylcholine (Roche) in various concentrations; prostigmine (Roche); and curare (crude Brazilian product). All these drugs were administered by injection into the abdominal aorta under the conditions described above, except atropine, which was injected into the external jugular vein in a dosage of 1 mg. per kg. body weight, being used with each administration of acetylcholine.

At the outset of each experiment involving picrotixin, one fourth to one third of the anesthetic dose of Nembutal was given as a supplementary to counteract the convulsant effect of the picrotoxin. In cases in which nikethamide reduced the anesthetic value of the barbiturate, such supplementary doses of Nembutal were also given.

RESULTS. I. *The effect of picrotoxin on indirectly stimulated muscle.* The action of picrotoxin was studied by its being injected into the abdominal aorta during indirect stimulation of the quadriceps femoris muscle. A 2% aqueous solution was used, the doses being between 0.2 and 1.0 mg. The frequency of stimuli varied between 100 per minute and 300 per second.

The action of picrotoxin is dependent on the rate of stimulation. It has no effect at all at frequencies of 100–150 per minute. At higher frequencies, on up to 32 per second, the responses are few and doubtful, there being an occasional slight depression. At higher frequencies, from 32 to 300 per second, picrotoxin always causes a depression of the muscular contraction, but this depression is small and of short duration. Figure 1 represents the results obtained in a typical experiment. One can observe in this figure that the depression is increased with increasing doses of picrotoxin.

II. *Effect of nikethamide on muscular contraction.* Nikethamide, differing from picrotoxin, has an intense action on muscular contraction. For greater clarity the discussion is subdivided.

A. *Indirect muscular stimulation.* Maximum stimuli were used with frequencies between 48 per minute and 500 per second. Effects were studied on both the quadriceps and the soleus muscles. The doses of nikethamide ranged between 0.125 and 0.0005 gms., those most used being 0.05 to 0.075 gms. In many experiments the minimum effective dose was found to be 0.0065 gm.

The action of nikethamide depends upon the frequency of stimulation. In sixteen experiments, using a frequency of excitation up to between 5 and 10 stimuli per second, there was an increase in the height of muscular contraction in twelve cases, a depression in two, and no response in two. The increase of muscular contraction is manifest in one of two ways; viz., either an augmenting of the contraction without effect on the base line of the graph, which is the more frequent observation, or, less commonly, the actual production of a supplementary contraction, noticeable in that the base line of the graph shows an ascent. (fig. 2). The latter type of reaction has been observed only occasionally and and only under these experimental conditions.

can be seen in fig. 3B, nikethamide can, in addition to depressing the amplitude of the first stage, depress even further the 3a stage and can exaggerate the phases of lack of transmission which are represented in stages 2 and 3b.

B. *Effect of nikethamide on the action of curare and prostigmine.* In these cases a frequency of stimulation fluctuating between 70 and 120 excitations per minute was used. In none of the experiments was a dosage of curare sufficient to necessitate artificial respiration. The doses of prostigmine were between 125 and 200 micrograms; nevertheless, it was at times necessary to repeat the dose in order to prostigminize the animal fully. Nikethamide was injected during the prostigminization and curarization and it was noted that this drug invariably reinforced the action of both prostigmine and curare as shown in figs. 4 and 5.

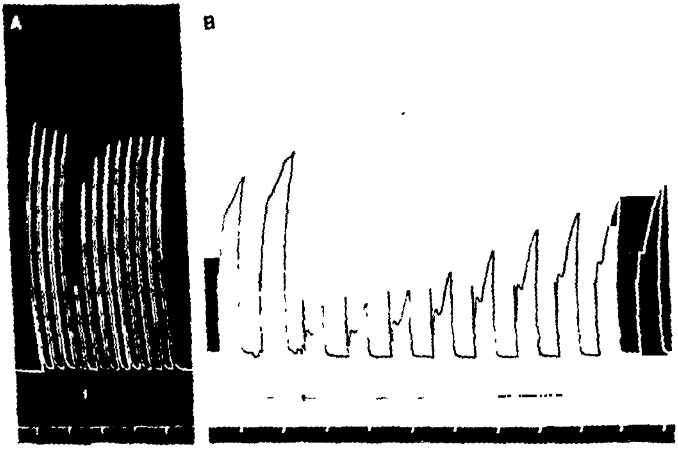


FIG. 3. DEPRESSANT ACTION OF NIKETHAMIDE ON THE FIRST STAGES OF NEUROMUSCULAR CONTRACTION

A. Cat anesthetized with Nembutal. Upper tracing: maximum stimulation of quadriceps through crural nerve with a frequency of 60 per second for 5 seconds and rest for 15 seconds. Upper line: intra-aortic injection of 75 mg. of nikethamide. Lower line: time in minutes.

B. Same animal as A. Upper tracing: maximum stimulation of soleus muscle through crural nerve with a frequency of 500 per second. Stimuli were of 15 seconds duration with rest periods of 15 seconds. When the muscle had developed sufficient tension, 25 mg. of nikethamide were injected intra-aortically, (signal on upper line). Lower line: time every half minute. For further details, see text.

C. *Effect of nikethamide on the response of muscle stimulated by the action of acetylcholine.* As the results described in Section B are contrary to the classical descriptions of the mode of action of curare and prostigmine (see Discussion) it was considered important to learn whether nikethamide was capable of increasing the sensitivity of a muscle in the presence of acetylcholine. It is known that a muscle is sensitized to the action of acetylcholine by preliminary denervation (Brown, Dale and Feldberg, 1936) and under these conditions a dose of only about $\frac{1}{100}$ the amount of acetylcholine as ordinarily required is capable effect of nikethamide on other stages of muscular contraction. The procedure was as follows: If a muscle be stimulated at a frequency of 60 per second for 3 to 5 seconds, there is obtained a contraction which represents exclusively the first stage. If these excitations be repeated at intervals of 15 to 30 seconds, a series of contractions of equal amplitude is obtained, each member of the series representing the first stage of neuromuscular transmission. The injection of nikethamide applied in the midst of one of these series of stimulations, as is shown in fig. 3A, produces an intense depression which can last for some time before the full reestablishing of the muscular contraction. If, on the other hand, the muscle is stimulated at a high frequency, for example 500 excitations per second, the first, second, 3a, 3b, 3c, and fourth stages appear. If these stimuli are of short duration, as 10 to 15 seconds, and if they are repeated every 10 to 15 seconds, there can be obtained after a time a series of contractions of equal amplitude in which each of the stages can be well visualized. Nevertheless, in other experiments these stages are summated and there appears a series of contractions which are quite difficult to interpret. If nikethamide is injected during these series of contractions two phenomena are apparent. First, when the stages are not summated in the muscular contractions the nikethamide causes the succeeding muscular contractions to develop only a very slight tension, as is shown in fig. 3B. Later the contractions show complete recuperation. Second, when the stages appear summated in the contractions, the nikethamide not only produces a depression with subsequent recuperation, but causes the stages of neuromuscular transmission to appear quite clearly. Moreover, as

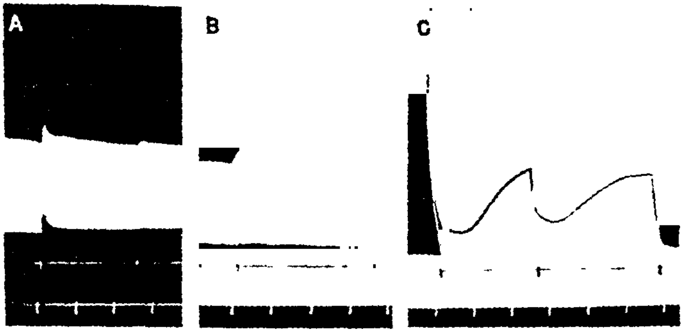


Fig. 2. Action of Nikethamide on the Contraction of Indirectly Stimulated Muscle

A. Cat anesthetized with Nembutal. Upper tracing: maximum stimulation of quadriceps indirectly at a frequency of 5 per second. Upper line: first signal, intra-aortic injection of 75 mg. of nikethamide; second signal, intra-aortic injection of 35 mg. of nikethamide. Lower line: time in minutes.

B. Cat anesthetized with Nembutal. Upper tracing: maximum stimulation of quadriceps indirectly at a frequency of 5 per second. Upper line: at the signal, intra-aortic injection of 75 mg. of nikethamide. Lower line: time in minutes.

C. Same animal as B. Upper tracing: maximum stimulation of quadriceps indirectly at a frequency of 350 per second. Upper line: first and last signal, onset and end of stimulation; middle signal, intra-aortic injection of 35 mg. of nikethamide. Lower line: time in minutes.

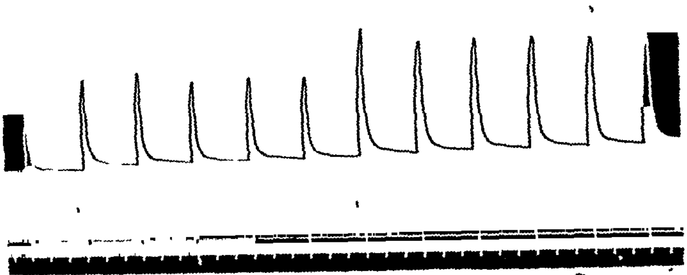


FIG. 6. REINFORCING ACTION OF NIKETHAMIDE ON MUSCULAR CONTRACTION PRODUCED BY ACETYLCHOLINE

Cat anesthetized with Nembutal. 1 mg. of atropine per kg. body weight. Sciatic nerve sectioned aseptically 10 days previously. Upper tracing: soleus muscle. Upper line: first signal, intra-aortic injection of 63 mg. of nikethamide; second signal, intra-aortic injection of 63 mg. of nikethamide. Middle line: each signal signifies the intra-aortic injection of 30 micrograms of acetylcholine dissolved in 0.30 cc. of distilled water. Lower line: time in minutes.

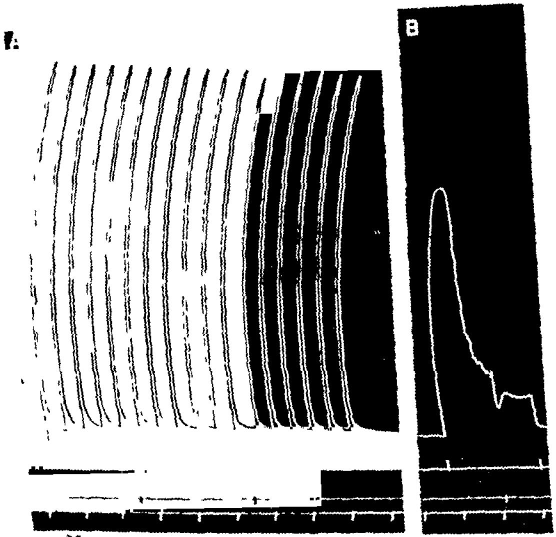
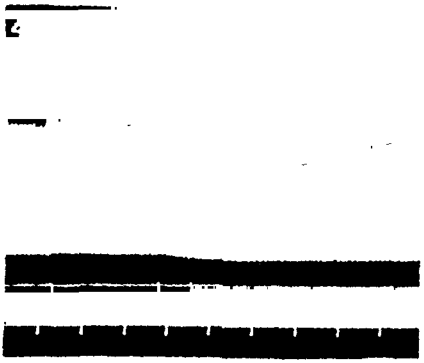


FIG. 7. ACTION OF NIKETHAMIDE ON THE CONTRACTION OF DIRECTLY STIMULATED MUSCLE

A. Cat anesthetized with Nembutal. Upper tracing: maximum contraction of soleus stimulated directly by means of a Harvard induction coil for 5 seconds every 25 seconds. Middle line: first signal, intra-aortic injection of 75 mg. of nikethamide; second signal, intra-aortic injection of 150 mg. of nikethamide. Lower line: time in minutes.

B. Same animal as in A. Upper tracing: maximum contraction of soleus stimulated directly by means of a Harvard induction coil. Upper line: first and second signal, onset and end of stimulation. Middle line: at the signal, intra-aortic injection of 63 mg. of nikethamide. Lower line: time in minutes.

stimuli applied for 5 seconds every 25 seconds, or, in some cases by the application of a tetanizing current persistently for a longer period. In the former instance, of producing a good contraction. If a certain quantity of the drug is injected every 2 to 4 minutes, there is obtained after a time a series of contractions of uniform amplitude. In order to obtain such a result the soleus was denervated aseptically 8 to 10 days prior to the experiment, and, after atropinization of the animal, 30 to 80 micrograms of acetylcholine in a volume of distilled water no greater than 0.30 cc. was injected into the abdominal aorta. When a series of uniform contractions was obtained the nikethamide was injected. In six cases out of seven the drug produced an important increase in the amplitude of muscular contraction. (In the seventh animal a satisfactory uniformity of amplitude of contraction was not obtained, so that it was not possible to observe distinctly the effect of the drug.) This reinforcement of the action of acetylcholine can last for some time as can be seen in fig. 6, and yet, during this reinforced response an additional dose of the nikethamide will cause an even further increase of amplitude (fig. 6).



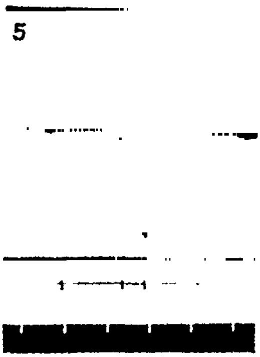


FIG. 4. REINFORCING ACTION OF NIKETHAMIDE ON THE EFFECT OF CURARE

Cat anesthetized with Nembutal. Upper tracing: maximum stimulation of quadriceps indirectly at a frequency of 100 per minute. Upper line: first signal (from left to right), intra-aortic injection of 50 mg. of nikethamide; second signal, intra-aortic injection of curare; third signal, intra-aortic injection of 50 mg. of nikethamide. Lower line: time in minutes.

FIG. 5. REINFORCING ACTION OF NIKETHAMIDE ON THE EFFECT OF PROSTIGMINE

Same cat as fig. 4, the effect of curare having worn off. Upper tracing: stimulation of quadriceps as in fig. 4. Upper line: first signal, (left to right), intra-aortic injection of 125 mg. of prostigmine; second signal, intra-aortic injection of 200 mg. of prostigmine; third signal, intra-aortic injection of 50 mg. of nikethamide. Lower line: time in minutes.

D. *Effect of nikethamide on the response of directly stimulated muscle.* If a muscle is stimulated directly not only are the muscle fibres stimulated but the nerve fibres are affected as well. Therefore, preliminary denervation of the muscle is necessary by section of its nerve. Three or four days after nerve section the degenerative phenomena are so advanced as to permit the muscular stimulation to be considered as genuinely direct. (Lissak, Dempsey and Rosenblueth, 1939). The soleus muscle, denervated 4 to 7 days prior to the experimentation, was used. Stimulation was obtained with maximum tetanizing

1936). That is, when an animal has received a dose of curare adequate to prevent muscular response on the stimulation of the motor nerve, an injection of eserine or prostigmine will permit the appearance of the muscular response to the nerve-conducted impulse.

The explanation of the antagonism between these drugs in the light of the chemical theory of nerve impulse transmission is as follows. Dale, Feldberg and Vogt (1936) have shown that the curarization of an animal does not impede or even quantitatively alter the liberation of acetylcholine on the indirect stimulation of the neuromuscular preparation. Rosenblueth and Morison (1937) believe that in order for acetylcholine to stimulate a muscle it must be present within an "effective range"; if the quantity should exceed this limit, reaching the paralytic threshold, instead of producing a greater stimulation of the muscle it produces a paralysis. On the other hand, if the quantity of acetylcholine does not reach the effective range, but remains below the excitatory threshold, there there is no contraction at all. These authors then explain the action of curare by suggesting that it raises the excitatory and paralytic thresholds. Then, according to them, the antagonistic action of eserine and curare is readily explained, since both eserine and prostigmine have a protective action toward acetylcholine by virtue of their property of inhibiting the activity of choline esterase, favoring an accumulation of the acetylcholine liberated during the nerve impulse. In other words, the function of eserine or of prostigmine might be explained by saying that each drug brings about a relative lowering of the above-mentioned thresholds.

Any substance, the effects of which are added favorably to those of eserine or or prostigmine, should decurarize the animal; that is, should cause the reappearance of the muscular contraction when a motor nerve is stimulated in a curarized animal.

Although the threshold theory of Rosenblueth and Morison serves quite well to explain a group of phenomena associated with the chemical transmission of the nerve impulse, it fails to give satisfactory explanation of the mode of action of nikethamide at the level of the neuromuscular junction. This drug produces a greater development of tension in the muscle stimulated indirectly at low frequencies (Section A and fig. 2); it produces a depression of muscular contraction when the muscle is stimulated indirectly at high frequencies (Section A and figs. 1 and 2); it increases the muscular contraction induced by acetylcholine (Section C and fig. 6); and it enhances the action of prostigmine (Section B and fig. 5). In the light of all of these reactions one could assume that the drug lowers the thresholds of acetylcholine, but if this is accepted there remains completely unexplained the fact that nikethamide enhances the action of curare (Section B and fig. 4).

If it is to be accepted that nikethamide lowers the thresholds of acetylcholine it is easy to explain the depression it produces in the first stage of muscular contraction (fig. 3A). On the other hand, it is quite difficult to explain the modifications resulting in the other stages; viz., 2, 3a, 3b, 3c. As has been pointed out above, nikethamide causes an accentuation of the stages of lack of contracwhen a series of uniform contractions were obtained nikethamide was injected. *Under these conditions nikethamide failed to have any effect, even when used* in double the usual doses (fig. 7.). On the other hand, when injected during a prolonged muscular contraction incited by a tetanizing current (The Harvard induction coil produces a high frequency, about 90 per second) nikethamide produces a depression in muscular contraction. (The nikethamide was always *injected in the descending phase of the curve of muscular tension*). When compared with the depressions obtained during indirect muscular contraction, this depression is of slight degree and of short duration (see fig. 7B).

DISCUSSION. I. *Action of picrotoxin.* As stated above, when picrotoxin is injected during muscular contraction it usually has no effect at all, or else it *depresses slightly the neuromuscular transmission.* This does not necessarily mean that picrotoxin could not have a more definite inhibitory effect if injected in larger doses. However, the doses which we did use were quite large, the greatest being 1 mg., and we did not feel that it was of value to study this drug further since its action on the neuromuscular junction was so slight.

Strychnine, metrazol, and picrotoxin are drugs which produce convulsions. The convulsions resulting from the action of strychnine and metrazol are quite similar, but those caused by picrotoxin are of a different nature (for details see Goodman and Gilman, 1941). Furthermore, the mode of action of these drugs on the neuromuscular junction differs; i.e., strychnine and metrazol produce inhibition of indirect muscular contraction by elevating the threshold of acetylcholine (Lanari and Luco, 1939; Salvestrini, Croxatto and Alonso, 1940). On the other hand, picrotoxin in doses up to 1 mg., which is the convulsant dose, has practically no action on indirect muscular contraction. Perhaps these differences in peripheral action have some relation to the fact that the types of convulsions produced by them are different.

II. *Action of nikethamide.* The experimental results described above lead one to think that the action of nikethamide takes place at the level of the neuromuscular junction. In Section D it is pointed out that nikethamide produces a slight depression when injected in the depressive phase of direct muscular contraction, that is, when there is muscular fatigue. However, when injected during a series of direct muscular contractions without muscular fatigue (see Section D), the nikethamide does not produce depression even when the dose is doubled. (Compare figs. 7A and 7B.) In our opinion this is only an apparent contradiction. In the whole animal, as were the animals used in this study, nikethamide produces an important hypertension by causing a vasoconstriction (see Goodman and Gilman, 1941). During the state of fatigue it is important that there be a good circulation in order to remove the metabolites. If during this state there be a vasoconstriction and thus a limitation of blood supply, the fatigue should increase. We believe that this might explain the production of depression by nikethamide in fatigued muscle when it has no effect on rested muscle.

The antagonistic action of eserine and prostigmine on the one hand and curare on the other is well known (for references see Rosenblueth, Lindsley and Morison, Nikethamide depresses the first stage of neuromuscular transmission (fig. 3A) and also depresses the amplitude of the contractions of a muscle stimulated indirectly every 15 seconds with a frequency of 500 stimuli per second. The first stages, namely 1, 2, 3a, 3b, and 3c, undergo a series of changes which are described in Section II A (fig. 3B). The action of curare (fig. 4) and the action of prostigmine (fig. 5) are reinforced by the action of nikethamide.

The contractions produced by acetylcholine in a muscle sensitized by denervation are reinforced by the effect of nikethamide. (Fig. 6).

Nikethamide does not alter the curve of the contraction of a directly stimulated muscle so long as there is not fatigue (fig. 7A), but depresses the curve in the presence of muscular fatigue. (Fig. 7B). This difference is discussed.

These results are analyzed in the light of our present knowledge of the chemical theory of nerve impulse transmission.

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tion (2 and 3b) and depresses the 3a stage. (Section A and fig. 3B.) According to Rosenblueth and Cannon (1940) the explanation of the effect on these first stages of neuromuscular contraction would be the following; stages 1; 3a and 3c would be identical and would be due to the liberation of acetylcholine; the first depression or lack of transmission stage 2, would be due to an inhibitory substance thus far unknown, and the second, 3b, to an excess of acetylcholine which would rapidly accumulate in the neuromuscular junction.

The injection of acetylcholine during the first stage only produces an exaggeration of stage 3b (Rosenblueth and Cannon) and does not appear to influence the others or the succeeding muscular contractions of a series of contractions obtained by a stimulation of 10 seconds followed by a rest of 10 seconds. If nikethamide lowered the threshold of acetylcholine it should be possible to observe an intense depression of stage 3b, since a lowering of threshold could not in any case compare with the excess of acetylcholine built up by the intra-arterial injection of 1 mg. of acetylcholine, as Rosenblueth and Cannon have been able to do without affecting the succeeding contractions. Such a depression does not occur. If, moreover, a relative lowering of threshold came about by a mechanism of inhibiting choline esterase it should be expected to affect more than one member of a series of contractions. This explanation does not correspond with observed phenomena if one compares these possibilities with the effects produced by prostigmine, a known inhibitor of choline esterase. In fact, according to Rosenblueth and Cannon, when prostigmine has been administered and the muscle is stimulated indirectly, frequencies of 30 to 60 stimuli per second are sufficient to produce the stages 1, 2, 3a, 3b, and 3c. If nikethamide lowered the threshold by a mechanism similar to that of prostigmine, it is evident that with a frequency of 500 stimuli per second, the stages under discussion would not be produced.

All of these considerations compel one to admit that the explanation of the mode of action of nikethamide cannot be similar to the already established explanations of the mode of action of these other drugs more commonly used in the study of nerve impulse transmission.

SUMMARY

In cats anesthetized with nembutal a study is made of the effect of picrotoxin on indirect contraction of the quadriceps femoris (Section I), the effect of nikethamide (coramine) on the indirectly stimulated soleus and quadriceps muscles (Section II, A), the effect of nikethamide on the response of muscle stimulated by the action of acetylcholine (Section II, C), and on the response of directly stimulated muscle (Section II, D).

When picrotoxin is injected during indirect stimulation of muscle it has either no effect at all or else only a slight depression of muscular contraction when the stimuli are of frequencies greater than 32 per second. (Fig. 1).

Nikethamide increases the development of tension of a muscle stimulated indirectly at low frequencies (fig. 2A and 2B) and produces a depression of muscular contraction when the stimuli are of higher frequencies (fig. 1 and 2C).

trodes were of silver with rubber insulation. In order to record the responses of the muscle, the femur was fixed with clamps and the tendon, isolated and divided, was fastened to the short end of a lever exercising traction on rubber bands and making kymographic tracings.

Drugs used in the study were as follows: Prostigmine (injectible solution, Roche) curare Merck or Brazilian (crude product), picrotoxin (Eimer and Amend) in 2% solution, strychnine sulfate Merck in 0.5% solution, and metrazol (pentamethylene tetrazol, Sanitas) in 10% solution. All solutions were made with distilled water. All these drugs were injected into the abdominal aorta below the inferior mesenteric artery, the contralateral iliac artery and the median sacral artery having been ligated. In some experiments the inferior mesenteric artery and some of the vertebral arteries were also ligated.

Since picrotoxin, strychnine and metrazol are convulsant drugs, a little prior to their application an injection of supplementary Nembutal, amounting to ¼ to ⅓ of the anesthetic dose, was injected intraperitoneally, thus convulsions were avoided in a large number of the experiments.

RESULTS. In all of the experiments a frequency of excitation stimuli varying between 96 and 170 per minute was used. It is well known that a muscle stimulated at these frequencies develops a certain tension which it maintains over a long period. (Del Pozo, 1942). Under these conditions it is very convenient to carry out studies with curare of prostigmine since a desirable degree of curarization or prostigminization can be obtained without the occurrence of fatigue phenomena which ordinarily might confuse the results. In this study there have not been used at any time doses of curare or prostigmine of sufficient strength to abolish completely the muscular contractions, except on one occasion when a complete curarization was obtained for a very short time, as may be seen in fig. 3.

A. *Effect of picrotoxin in the action of prostigmine and of curare.* Picrotoxin, when injected into the abdominal aorta during the action of prostigmine or of curare, has very little if any effect. As seen in fig. 1, there is in the case of curare a scarcely perceptible change, which seems to be a reinforcement effect. It cannot be said that any more perceptible change was obtained when the largest doses of the experiment (1 mg.) were used than when the smallest dose was given (0.6 mg.). In any case, the reinforcing effect of picrotoxin on curare is extremely small, if it exists at all.

It is even more difficult to point out any effect of picrotoxin on the action of prostigmine, either of reinforcement or of inhibition. If it has any action at all, it would seem to be one of interference with the phenomenon of spontaneous deprostigminization.

B. *Effect of strychnine on the action of prostigmine and of curare.* In contrast to the findings in Section A, strychnine does have a definite effect on the action of both curare and prostigmine, and a similar effect on the two. In all cases there was reinforcement of the action of either drug for a few seconds (fig. 2). Immediately after this reinforcement there occurs a moderate decurarization (2A) or a very marked deprostigminization (fig. 2B and 2C). All of these results were more intense when larger doses of strychnine (2 mg.) were used than when smaller ones (0.75 mg.) were given.

C. *Effect of metrazol on the action of prostigmine and of curare.* When metrazol is injected into the abdominal aorta during the action of prostigmine, it reinforces to a slight degree the action of the latter drug, (fig. 4), an observation confirming

EFFECT OF PICROTOXIN, STRYCHNINE AND METRAZOL (PENTAMETHYLENETETRAZOLE) ON THE ACTION OF PROSTIGMINE AND CURARE ON THE NEUROMUSCULAR JUNCTION

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In a study on the effect of magnesium sulfate on the superior cervical ganglion, Huidobro and Luco (1939) found that this drug caused a diminution in the effectiveness of prostigmine and of curare. This curious property of magnesium sulfate is not manifest when this drug is applied to the neuromuscular junction (Luco, Pichard, and Huidobro, 1940). Salvestrini, Croxatto and Alonso (1940) obtained an opposite effect with metrazol in their studies on the neuromuscular junction, finding that this drug potentiates the action of prostigmine and of curare. On the other hand Kahlson and Peil (1937) have shown that metrazol is capable of restoring respiratory function which has been stopped by curare. Although there are other substances which show these paradoxical effects on the action of curare and prostigmine, as in the case of nikethamide (Huidobro and Jordan, 1945), it seemed of interest to us to reinvestigate the effects of metrazol since there is a contradiction between the findings of Kahlson and Peil on the one hand and Salvestrini, Croxatto and Alonso on the other.

As Salvestrini, Croxatto and Alonso (1940) have indicated that there is a similarity between the action of metrazol on the superior cervical ganglion and its action on skeletal muscle as regards its influence on the action of strychnine on these two structures (Lanari and Luco, 1939), we believe it is important to learn whether such a similarity exists in its influence upon the action of curare and prostigmine.

We have chosen to include observations on the action of picrotoxin in this study because of its pharmacological similarity to metrazol and strychnine, all three being central nervous system stimulants and convulsants. Picrotoxin apparently does not have any noticeable effect on the neuromuscular junction (Huidobro and Jordan); however, it has been noted in this department (Huidobro and Valenzuela) that some substances which seem not to have any effect on the neuromuscular junction were actually slightly influential on this junction when the animal had been injected with curare or prostigmine. This fact leads us to include picrotoxin in the present study despite previous negative findings with this drug.

Method. The animals used were cats weighing 2 to 3 kgs., anesthetized with sodium pentobarbital (Nembutal, Abbott: 0.033 Gm. dissolved in 1 cc. of 25% urethane, per kg. body weight, intraperitoneally). A tracheal cannula was inserted so as to facilitate artificial respiration if necessary. The studies were made on the quadriceps femoris muscle, stimulated with maximal excitations through the crural nerve, previously isolated and divided. Stimuli were discharged from condensers controlled with vacuum tubes. Elecdescribed (Huidobro and Jordan, 1945). In reality, picrotoxin has only a very slight effect, if any at all, on indirectly stimulated muscle and on the action of prostigmine and of curare. Nevertheless, it does seem to reinforce the action of curare and prostigmine (see Section A, Results), the drug thus seeming to be feebly curarizing and feebly prostigminizing, the latter being particularly hard to demonstrate because of the impossibility of giving a satisfactory dose of prostigmine in the control experiments. This difficulty stems from the fact that prostigmine is eliminated very slowly and its effects are cumulative. If it is true that picrotoxin is curarizing and at the same time prostigminizing this fact is difficult to explain by our present knowledge in the light of the chemical theory of the transmission of nerve impulses. Indeed, for some time it has been known that curare and prostigmine are antagonistic (for references see Rosenblueth, Lindsley and Morison, 1936). The action of curare can be explained as an elevating of the threshold of acetylcholine (Rosenblueth and Morison, 1937), prostigmine on the other hand, acts by its anti-choline-esterase power, lowering the paralytic threshold of acetylcholine (Rosenblueth and Morison, 1937. For more complete discussion see Huidobro and Jordan, 1945).

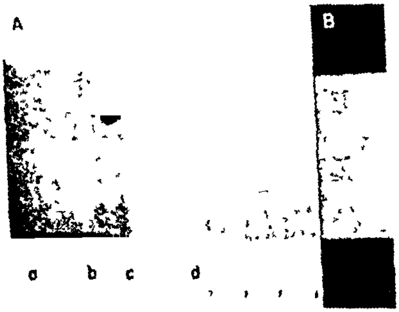

FIG 3 DECURARIZING ACTION OF METRAZOL

A Cat anesthetized with Nembutal Upper tracing quadriceps femoris muscle excited indirectly with maximum stimuli at a frequency of 100 per minute Upper line a, intra-aortic injection of 25 mg of metrazol, b and c, intra aortic injection of curare, d, intra aortic injection of 25 mg of metrazol Lower line time in minutes

B Eleven minutes after A.

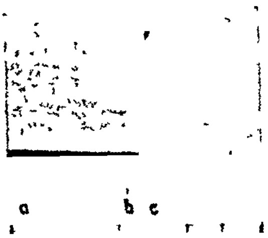

FIG 4 REINFORCEMENT OF THE ACTION OF PROSTIGMINE BY METRAZOL CAT ANESTHETIZED WITH NEMBUTAL

Quadriceps femoris muscle excited indirectly with maximum stimuli at a frequency of 140 per minute Upper line a, intra aortic injection of 30 mg of metrazol, b, intra aortic injection of 225 micrograms of prostigmine, c, intra aortic injection of 30 mg of metrazol Lower line time in minutes

B *Strychnine* Lanari and Luco (1939) have shown that strychnine has a depressant action on skeletal muscle and on the superior cervical ganglion Their explanation for this phenomenon is that strychnine elevates the acetylcholine threshold Thus, it may be said that strychnine and curare are analagous in this sense Bolly and Bacq (1938) observed that strychnine raised the threshold of acetylcholine Lanari and Luco, on the other hand, indicate that a small dose the findings of Salvestrini, Croxatto and Alonso (1940). In contrast to the observations of these writers, we find that when metrazol is injected into the abdominal aorta, it produces a decided decurarization as shown in fig. 3. This

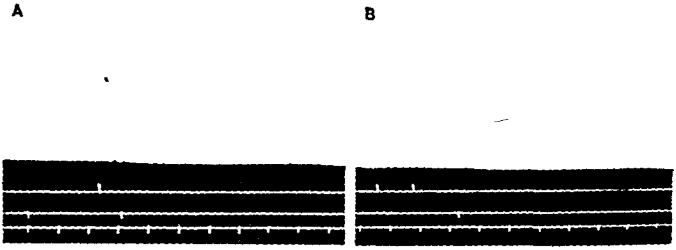

FIG. 1. EFFECT OF PICROTOXIN ON THE ACTION OF PROSTIGMINE AND OF CURARE

A. Cat anesthetized with Nembutal. Upper tracing: quadriceps femoris muscle excited by maximum stimuli through the crural nerve with a frequency of 120 per minute. Signal on upper line: intra-aortic injection of 225 micrograms of prostigmine. Signal on middle line: intra-aortic injection of 0.6 mg. of picrotoxin. Lower line: time in minutes.

B. Same animal as in A. Upper tracing: stimulation of quadriceps femoris muscles under same conditions as in A, 11 minutes later. Signal on upper line: intra-aortic injection of 0.6 mg. of picrotixin. Lower line: time in minutes.

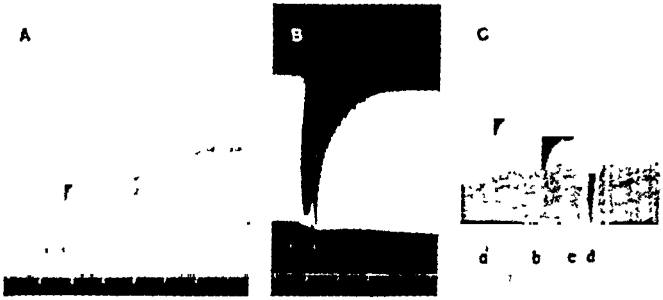

FIG. 2. EFFECT OF STRYCHNINE ON THE ACTION OF CURARE AND OF PROSTIGMINE

A. Cat anesthetized with Nembutal. Upper tracing: maximum indirect stimulation of quadriceps femoris muscle at a frequency of 160 per minute. Upper line: first signal, intra-aortic injection of curare, second signal, intra-aortic injection of 0.75 mg. of strychnine. Lower line: time in minutes.

B. Cat anesthetized with Nembutal. Upper tracing: quadriceps femoris muscle stimulated under the same conditions as in A. Upper line: first signal, intra-aortic injection of 275 micrograms of prostigmine, second signal, intra-aortic injection of 1.5 mg. of strychnine. Lower line: time in minutes.

C. Cat anesthetized with Nembutal. Upper tracing: quadriceps femoris muscle stimulated indirectly at a frequency of 120 per minute. Upper line: a., intra-aortic injection of 300 micrograms of prostigmine; b., intra-aortic injection of 150 micrograms of prostigmine, c., intra-aortic injection of 200 micrograms of prostigmine; d., intra-aortic injection of 2 mg. of strychnine. Lower line: time in minutes.

is a confirmation of the findings of Kahlson and Peil (1937). These effects have been definite and constant in the eight animals studied, the doses of metrazol varying between 15 and 30 mg. The drug was injected into the abdominal aorta under the conditions described above (see Methods).

DISCUSSION. A. *Picrotoxin.* The results obtained concur with those already strychnine has a definite inhibitory action on choline esterase. This phenomenon stands in contradiction to the observations reported above, since strychnine, except for a very brief period of reinforcement of prostigmine, has a definite deprostigminizing effect.

Picrotoxin, strychnine and metrazol are stimulants of the central nervous system and the three produce convulsions. In the neuromuscular junction the three drugs act as quite different substances (see Results, Section A, B, and C). Picrotoxin has practically no effect; strychnine has a very curious effect on the action of curare and of prostigmine; and metrazol is depressant in that it evidently lowers the acetylcholine threshold. These marked differences suggest that the chemical transmission in the synapses in the central nervous sytem must take place under circumstances differing from those which facilitate it in the neuromuscular junction.

SUMMARY

Studies are carried out in cats anesthetized with Nembutal in order to observe the effects on the indirectly stimulated quadriceps muscle when the animal has received prostigmine under curarization. The following observations are made.

Picrotoxin has practically no effect. What little it does have seems to be a feeble reinforcement of the effect of prostigmine and of curare (fig. 1 and Section A of Results).

Strychnine has a complex reaction. For a few seconds it reinforces the action of curare and of prostigmine and then produces a moderate decurarization or a violent deprostigminization (fig. 2 and Section B of Results).

Metrazol weakly reinforces the action of prostigmine and behaves as a decurarizing drug (fig. 3 and Sec. C of Results).

The behaviour of these three substances is discussed in the light of our present knowledge of the theory of neural transmission. The conclusion is drawn that the behavior of picrotoxin and strychnine cannot be explained by the classical theory of the two thresholds of acetylcholine, whereas metrazol can be looked upon as a neuromuscular depressant in that it elevates the acetylcholine thresholds.

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of strychnine (0.25 to 0.50 mg.) increases the response of indirectly stimulated muscle which has previously been depressed by strychnine. They report similar results with somewhat larger doses (0.50 to 1 mg.) in the superior cervical ganglion. The results described in Section B appear to be in contradiction to the reports of these authors, since in our findings strychnine exaggerates the action of prostigmine, although it is true that the reinforcement is of short duration and is followed by an intense deprostigminization. In their studies, Lanari and Luco stimulated the preganglionic fibers for 2 seconds with tetanic current and, in the case of muscle, stimulated the nerve for 10 seconds tetanically, and allowed the preparation to rest, in the case of the ganglion, for one minute and in the case of muscle for three minutes. Therefore it is possible that their injection of prostigmine was not made at the exact moment in which the drug might have been reinforced by the strychnine.

Since Lanari and Luco have showed that strychnine does not activate muscle directly and only has influence on muscle through the neuromuscular junction, it is not possible to explain the similarity of the effect of strychnine on the action of curare and on the action of prostigmine, as set forth in Section A.

C. *Metrazol.* We find that metrazol has a decurarizing action, a confirmation of the observations of Kahlson and Peil (1937) and a contradiction of the statements of Salvestrini, Croxatto, and Alonso. We felt that it would be of interest to repeat some of the experiments of these authors in order to see whether the discrepancies might lie in differences in the varying nature of the drug employed.

The inhibitory action of metrazol on muscular contraction depends on the frequency of stimulation. The drug fails to have a depressant action on the contraction of a muscle stimulated at a low frequency, between 90 and 170 per minute, (see fig. 3 and 4), thus failing to conform to the observations of Salvestrini, Croxatto, and Alonso. If high frequencies are used, above 25 per second, metrazol produces an intense depression of muscular contraction, very often without any recovery, thus conforming with the claims of the above mentioned authors. Furthermore, as these workers state, we find that if metrazol is injected during a series of stimulations of high frequency and short duration, applied at regular intervals of time, there does come about a depression of contractions with complete failure to regain the original amplitude.

If we accept the idea that metrazol does not act directly on muscle (Salvestrini, Croxatto and Alonso, 1940) either to depress it at high frequencies or to reinforce the action of prostigmine or to produce decurarization, it is necessary to suppose that the drug has a depressant action on the neuromuscular junction, since it is capable of lowering the acetylcholine threshold. In other words, when metrazol is injected during indirect muscular contraction it produces a situation in which the quantity of acetylcholine liberated in the nerve ending is not sufficient for the chemical transmission of the impulse, since the amount is in excess of the paralytic threshold (Rosenblueth and Morison, 1937).

If, then, we accept the idea that metrazol lowers the acetylcholine threshold, we must assume that it does so through a mechanism other than that generally recognized, because metrazol does not have any influence upon the potency of choline esterase (Sepulveda and Croxatto, 1940). Nachmansohn affirms that

Pharmacological investigations with pure erythrophleum alkaloids have been relatively few. Santi and Zweifel (14) have tested their toxicity in frogs, rats, and rabbits. Brief reports have been published of their local anesthetic effect (15) and their action upon the isolated frog heart (16), the frog heart in situ (17), the isolated rabbit heart (18), the blood vessels of the hind legs of the frog and of the rabbit's ear (19), the blood pressure and respiration of the rabbit (20), the isolated non-pregnant uterus (21), and isolated rabbit intestine (22).

Chen, Hargreaves, and Winchester (23) have established the minimal lethal and emetic dose in the cat and the minimal systolic dose in the frog.

This report is a description of the action of some of the erythrophleum alkaloids upon the isolated mammalian heart in the form of the heart-lung preparation of the dog. A further study of the cardiac action of these alkaloids seemed of interest because the chemical structure has now been sufficiently clarified to show the difference between these compounds and the cardiac glycosides, as well as a group of other alkaloidal substances, the veratrum alkaloids, the positive inotropic action of which has recently been investigated in greater detail in this laboratory (24, 25, 26).

The alkaloids used in our experiments were: cassaine hydrochloride, nor-cassaidine hydrochloride, erythrophleine sulfate, and coumingine hydrochloride. In addition, a few observations were made on the action of erythrophleic acid. The substances were put at our disposal by Dr. K. K. Chen, who had obtained the cassaine hydrochloride, nor-cassaidine hydrochloride, and coumingine hydrochloride from Dalma, the erythrophleine sulfate from E. Merck, Darmstadt, Germany, and the erythrophleic acid from Dr. A. R. Todd at the University of Manchester, Manchester, England.

METHODS. Twenty-four experiments were performed on the heart-lung preparation. The heart-lung dogs weighed from 9 to 14 kgm. They were anesthetized with 35 mgm. of sodium pentobarbital (nembutal)[1] per kgm. given intraperitoneally. Defibrinated blood was used to supply the heart-lung preparation. The methods of recording changes in heart action were the same as those described by Krayer and Mendez (24). Heart failure was either spontaneous or induced by the injection of sodium pentobarbital. All injections, both of sodium pentobarbital and of the erythrophleum alkaloids, were made into the blood before it entered the venous reservoir. The erythrophleum alkaloids were dissolved in a concentration of 1:1000 in 0.9 per cent sodium chloride solution. Erythrophleic acid was dissolved in a concentration of 1:500 in 0.9 per cent sodium chloride solution and was neutralized with sodium bicarbonate. Each solution was made just before the experiment in which it was used. Heart rates were counted either directly or from electrocardiographic tracings recorded with a Grass ink-writing oscillograph.

RESULTS. All the alkaloids investigated by us have (1) a characteristic positive inotropic effect responsible for the ability of the alkaloids to improve the work capacity of the heart; (2) an influence upon the rate of the heart characterized especially by irregularities. The alkaloids differ considerably, however, in the ratio of the minimal dosages leading to irregularities and those which will

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[1] The sodium pentobarbital used in this work was generously supplied by Abbott Laboratories, North Chicago, Illinois.

THE ACTION OF THE ERYTHROPHLEUM ALKALOIDS UPON THE ISOLATED MAMMALIAN HEART*

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In 1875–76 Gallois and Hardy (1, 2) isolated from the bark of *Erythrophleum guineense* an alkaloid which they called erythrophleine. Several other related alkaloids were found in the bark of *Erythrophleum guineense* (3) and in the bark of *Erythrophleum couminga* by Dalma. The knowledge of the chemistry of the erythrophleum alkaloids has made some progress since 1935 especially as a result of the work of Dalma, Ruzicka, Blount and their collaborators (4–11). Recently Craig (12) has summarized the status of our present chemical knowledge on these compounds.

The erythrophleum alkaloids are esters of dimethylaminoethanol or methylaminoethanol with tricyclic acids of probably diterpenoid nature, which on dehydrogenation yield 1,7,8-trimethylphenanthrene. On acid hydrolysis the alkaloids split according to the following formulae:

$C_{21}H_{39}O_5N$ Erythrophleine	$\longrightarrow$	$C_{21}H_{32}O_5$ Erythrophleic Acid	+	$CH_3{-}NH{-}CH_2{-}CH_2OH$ methylaminoethanol
$C_{24}H_{39}O_4N$ Cassaine	$\longrightarrow$	$C_{20}H_{30}O_4$ Cassainic Acid	+	$(CH_3)_2{-}N{-}CH_2{-}CH_2OH$ dimethylaminoethanol
$C_{24}H_{41}O_4N$ Cassaidine	$\longrightarrow$	$C_{20}H_{32}O_4$ Cassaidinic Acid	+	$(CH_3)_2{-}N{-}CH_2{-}CH_2OH$ dimethylaminoethanol
$C_{29}H_{47}O_6N$ *Coumingine*	$\longrightarrow$	$C_{25}H_{38}O_6$ *Couminginic* Acid	+	$(CH_3)_2{-}N{-}CH_2{-}CH_2OH$ *dimethylaminoethanol*

Cassainic acid has one carbonyl group, one hydroxyl group, and one double bond in α-β position to the carboxyl group. Erythrophleic acid has in addition one methoxyl group. Cassaidinic acid differs from cassainic acid in that it has an additional hydroxyl group instead of the carbonyl group. Couminginic acid is a derivative of cassainic acid in which the nuclear hydroxyl group is esterified with β-hydroxy*iso*valeric acid. No chemical information on nor-cassaidine except the empirical formula (3) $C_{23}H_{41}O_5N$ could be found in the literature.

Since Gallois and Hardy described systolic arrest of the heart of the frog resulting from erythrophleine, the erythrophleum alkaloids have been considered heart poisons and pharmacologically related to the cardiac glycosides. The older pharmacological literature has been exhaustively reviewed by Santi (13).

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* This work was supported in part by a grant from Eli Lilly and Company and in part by a grant from the William W. Wellington Memorial Research Fund.

increase in output and little rise in right auricular pressure, although the output values were somewhat lower than the control values.

As is the case with the cardiac glycosides and the veratrum alkaloids, the onset of the action is not abrupt, but gradual. It becomes noticeable within one to two minutes after cassaine hydrochloride and nor-cassaidine hydrochloride, and within three minutes after erythrophleine sulfate. Improvement of contractility reaches its maximum within a period of five to fifteen minutes, as can be seen from the changes in ventricular volume (fig. 1) and venous pressure (fig. 1 and 2) and from the change in systemic output (fig. 2). The duration of action of a single nearly maximally effective positive inotropic dose (not leading to disturbances of rhythm) is approximately 45 minutes. In the experiment of figure 1, the diastolic volume had returned to its original level 45 minutes after the injection of 0.5 mgm. cassaine hydrochloride, while in the experiment of figure 2, the increased work capacity was still evident 37 minutes after the administration of 0.25 mgm. nor-cassaidine hydrochloride.

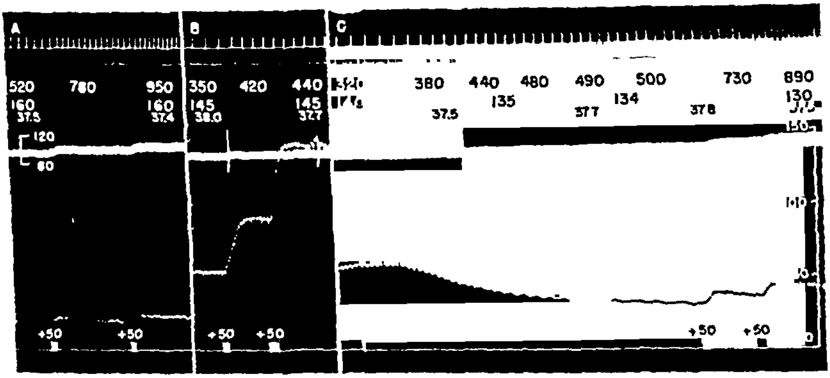

FIG. 2. EXPERIMENT NO. 10. ACTION OF NOR-CASSAIDINE HYDROCHLORIDE UPON THE HEART IN SODIUM PENTOBARBITAL FAILURE

Heart-lung preparation. Weight of heart-lung dog, 10 kgm. Ventricular weight, 47 grams. Blood volume approximately 630 cc. during A, 450 cc. during B and C. Arterial resistance 66 mm. of mercury. Tracings from top to bottom: systemic output, each signal indicating 100 cc., time in 10 second intervals, arterial pressure (scale on left in mm. of mercury), right auricular pressure (scale on right in mm. of water). The horizontal rows of figures indicate, from top to bottom: systemic output in cc. per minute, heart beats per minute, temperature of the blood in degrees centigrade. At +50, the level of the venous reservoir was increased by 50 millimeters. Between A and B, during a time lapse of 18.5 minutes, 140 mg. of sodium pentobarbital was injected. Between B and C, 3 minutes' interval. During these intervals, the original inflow level was restored. At the first signal in C, 0.25 mgm. of nor-cassaidine hydrochloride was injected.

The pulmonary arterial pressure of normal heart-lung preparations is not changed by positive inotropic doses of cassaine, nor-cassaidine, or erythrophleine. However, the elevated pulmonary arterial pressure of the failing heart-lung preparation is reduced by such doses. The marked decrease in diastolic ventricular volume of the failing heart, therefore, is due in part to the change in pulmonary pressure with its marked influence upon the size of the right ventricle.

lead to a positive inotropic effect. On this basis, nor-cassaidine, cassaine, and, to a lesser degree, erythrophleine, differ sharply from coumingine.

I. THE POSITIVE INOTROPIC ACTION; THE EFFECT UPON PULMONARY PRESSURE AND UPON CORONARY FLOW. A. *Nor-cassaidine, cassaine, and erythrophleine. The positive inotropic effect* of the alkaloids is apparent from figure 1 and figure 2. In the normal heart (see fig. 1) the outstanding change is a decrease in diastolic ventricular volume with no marked change in systemic output. The lack of a change in heart rate and pulmonary arterial pressure indicates that the change in heart volume is due to the inotropic action of the alkaloid.

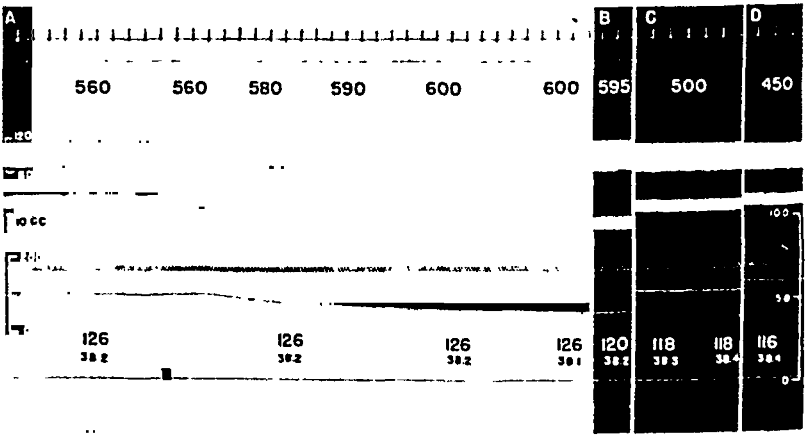

FIG. 1. EXPERIMENT NO. 5. ACTION OF CASSAINE HYDROCHLORIDE UPON HEART VOLUME
Heart-lung preparation. Weight of heart-lung dog, 10.6 kgm. Ventricular weight, 114 grams. Approximate blood volume, 550 cc. Arterial resistance not recorded. Tracings from top to bottom: systemic output, each signal indicating 100 cc.; time in 10-second intervals; arterial pressure (scale on left in mm. of mercury); heart volume (scale on left below the tracing in 10 cc.); pulmonary arterial pressure (scale on left in mm. of water); right auricular pressure (scale on right in mm of water). The horizontal rows of figures indicate, from top to bottom: systemic output in cc. per minute, heart beats per minute, and temperature in degrees centigrade. At signal in A, 0.5 mgm. of cassaine hydrochloride was given. B, 10 minutes after A. C, 30 minutes after B. D, 9 minutes after C.

As with the cardiac glycosides and veratrum alkaloids, work capacity changes without marked change in rate can be more strikingly demonstrated in the failing heart, whether the failure is spontaneous or induced by the administration of barbiturates. In the experiment of figure 2, heart failure was induced by sodium pentobarbital, and the limit of competency, as shown by the response to increase in venous supply, had been greatly reduced. From figure 2 B it appears that the heart was unable to increase its systemic output above 440 cc. per min. The administration of 0.25 mgm. nor-cassaidine hydrochloride increased the systemic output under basal conditions of blood supply to 96 per cent of its original value (from 320 cc. to 500 cc. per min.); and the subsequent increase in blood supply showed that the heart responded like the normal heart with marked

TABLE 1

EXP. NO.	ORDER OF INJECTION*	VENTRICLE WT.	DOSE		INOTROPIC EFFECT	CHRONOTROPIC EFFECT		REMARKS
			Mgm.	Mgm./L blood		Type§	Onset	
Cassaine Hydrochloride								
		gm.					min.	
1	A	79	0.1	0.17	none	none		PBF†
1	B	79	0.2	0.49	positive	none		13 min. after A
2	A	91	0.25	0.48	positive	none		PBF
3	A	77	0.3	0.33	none	none		normal heart
4	A	76	0.5	0.83	positive	none		PBF
5	A	114	0.5	0.93	positive	none		slight SF‡
6	A	106	0.5	0.91	positive	none		SF
3	B	77	0.6	0.74		v. tachycardia	4	51 min. after A
5	B	114	0.7	2.08	positive	none		56 min. after A
1	C	79	0.75	2.0	positive	none		15 min. after B
7	A	94	0.8	1.0	positive	slowing, a. v. block	31	PBF
8	A	73	1.0	2.0	positive	slowing	10	normal heart
Nor-cassaidine Hydrochloride								
9	A	92	0.15	0.39	positive	none		PBF
10	A	47	0.25	0.56	positive	none		PBF
11	A	86	0.25	0.42	positive	none		PBF
12	A	105	0.25	0.38	positive	none		slight SF
13	A	94	0.5	0.53	positive	transient extrasystoles	25	PBE
10	B	47	0.5	1.39		v. tachycardia	2	38 min. after A
12	B	105	0.55	0.86		v. tachycardia	3	25 min. after A
Erythrophleine Sulfate								
14	A		0.25	0.50	none	none		PBF
14	B		0.25	0.50	positive	none		5 min. after A
15	A	117	0.3	0.54	positive	none		PBF
16	A	122	0.5	0.89	positive	none		PBF
17	A	64	0.5	1.1	positive	none		slight SF
18	A	85	0.5	0.77	positive	v. tachycardia	23	PBF; irreg. when Morawitz cannula removed
15	B	117	0.5	0.89	positive	slowing	11	50 min. after A
14	C		0.5	1.0		v. tachycardia	12	15 min. after B
19	A	166	1.0	0.95		v. tachycardia	8	PBF

*A = first, B = second, C = third injection.

† = Pentobarbital failure.

‡ = spontaneous failure.

§ a. = auricular, v. = ventricular.

The coronary sinus outflow was measured in four experiments with cassaine, in three experiments with nor-cassaidine, and in one experiment with erythrophleine. Doses of the alkaloids which do not cause irregularities cause no significant changes, although a tendency to decrease in coronary flow is apparent. A definite reduction in coronary sinus outflow occurs with doses leading to irregularities (see fig. 3). In the experiment of figure 3, coronary sinus outflow decreased 37 per cent after 1 mgm. of cassaine hydrochloride. There was no important change in mean arterial pressure or rate to account for this change. The sinus outflow again increased with the appearance of irregularities which were precipitated by the attempt to test the response of the heart to increased blood supply. As is usually the case if coronary resistance increases in the normal heart, there was a shift of blood into the systemic circulation and the total output in this experiment did not significantly change until irregularities intervened.

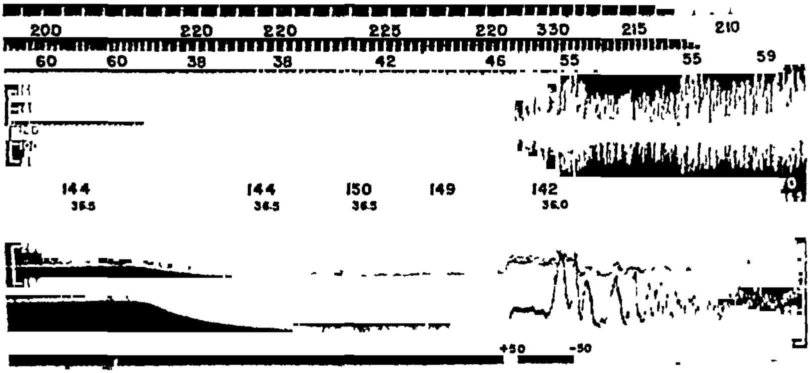

FIG 3 EXPERIMENT NO 8 ACTION OF A TOXIC DOSE OF CASSAINE HYDROCHLORIDE UPON THE NORMAL HEART

Heart lung preparation *Weight of heart lung dog, 9 8 kgm.* Ventricular weight, 73 grams Approximate blood volume, 520 cc Arterial resistance, 86 mm. of mercury. Tracings from top to bottom systemic output, each signal indicating 100 cc.; coronary sinus outflow, each signal indicating 0 2 cc , time in 10 second intervals, arterial blood pressure (scale on left in mm of mercury), pulmonary arterial pressure (scale on left in mm. of water), right auricular pressure recorded from inferior vena cava (scale on right in mm. of water) Horizontal rows of figures from top to bottom systemic output in cc per minute; coronary sinus outflow in cc per minute, heart beats per minute, temperature in degrees centigrade At +50, the level of the venous reservoir was raised by 50 millimeters. At −50, the original inflow level was restored At the first signal, 1 0 mgm. of cassaine hydrochloride was injected

The dosage range of the alkaloids employed in our experiments can be seen from table 1. The relative potency of the three alkaloids with regard to their positive inotropic action decreases in the following order: nor-cassaidine, cassaine, erythrophleine On a molar basis cassaine is only about one-half as potent as nor-cassaidine in leading to complete restoration of the performance of

A. *Cardiac slowing.* Cardiac slowing has been observed in experiments 7 and 8 with cassaine, in experiment 15 with erythrophleine, and in experiment 20 with coumingine.

The most striking direct effect upon the cardiac pacemaker was seen in experiment 20. About 18 minutes after the injection of coumingine, the heart rate decreased suddenly from 132/min. to 78/min. and remained slow throughout the rest of the experiment (25 minutes). There was no auricular-ventricular dissociation. Cardiac slowing without auricular-ventricular dissociation was also observed in experiment 15 after an injection of erythrophleine. Since electrocardiograms were not recorded in either of these experiments, we do not know whether an increased P-R interval accompanied the cardiac slowing.

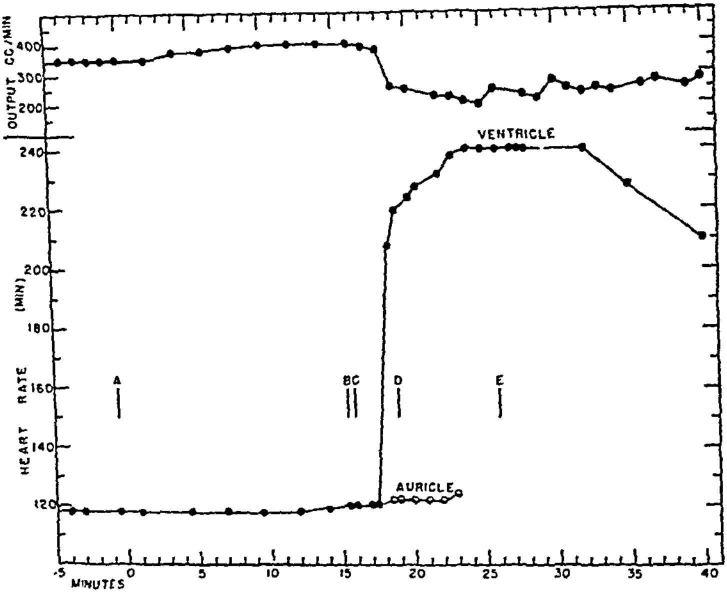

FIG. 4. EXPERIMENT NO. 23. ACTION OF COUMINGINE HYDROCHLORIDE UPON THE SYSTEMIC OUTPUT AND THE RATE OF THE NORMAL HEART

Heart-lung preparation. Weight of heart-lung dog, 11.2 kgm. Ventricular weight, 88 grams. Arterial resistance, 84 mm. of mercury. The temperature of the blood varied from 36.3°C. to 37.1°C. The heart beats per minute were counted from electrocardiographic tracings. At the point 0 on the time scale, 0.25 mgm. of coumingine hydrochloride was injected. The vertical lines labeled A, B, C, D, and E indicate the times at which the corresponding electrocardiograms in figure 5 were recorded.

The mechanism of the cardiac slowing in experiment 8 (fig. 3) is not clear. Experiment 7 will be discussed in detail in Section B.

Auricular asystole occurred in experiments 22 and 23 after doses of 1.0 mgm. of coumingine hydrochloride, in experiment 19 after a dose of 1.0 mgm. of erythrophleine sulfate, and in experiment 7 after a dose of 0.8 mgm. of cassaine hydrochloride.

B. *Action upon the conduction system.* A clear action of an erythroph-

TABLE 1—*Continued*

EXP. NO.	ORDER OF INJECTION*	VENTRICLE WT.	DOSE		INOTROPIC EFFECT	CHRONOTROPIC EFFECT		REMARKS
			Mgm.	Mgm./L blood		Type§	Onset	
Coumingine Hydrochloride								
		gm.					*min.*	
20	A	92	0.1	0.16	very slightly positive	slowing	18	PBF
21	A	82	0.2	0.33	positive	v. tachycardia	17	slight SF
22	A	80	0.2	0.29	positive	v. tachycardia	16	PBF
23	A	88	0.25	0.35	positive	v. tachycardia	17	normal heart
23	B	88	1.0	1.75		a. asystole v. fibrillation	6	42 min. after A
22	B	80	1.0	1.38		a. asystole v. fibrillation	5	75 min. after A

the failing heart, and erythrophleine is less than one-half as potent as nor-cassaidine. The number of experiments which we were able to make with erythrophleine sulfate does not make it possible to estimate the relative potency more precisely, but it appears that full restoration of the performance of the failing heart is difficult with this alkaloid as the doses required for this come close to those leading to changes in heart rate.

B. *Coumingine.* Coumingine hydrochloride causes a positive inotropic action in the heart-lung preparation at a dosage level below that of the other three alkaloids (see table 1). It was not possible, however, to effect a complete improvement of the failing heart with coumingine, as the doses used, while far from sufficient to restore the work capacity of the heart to normal, already caused a marked change in heart rate or cardiac irregularities. As with erythrophleine sulfate, the positive inotropic effect of coumingine hydrochloride starts after a latency period of about three minutes; that is, it develops more slowly than with cassaine hydrochloride and nor-cassaidine hydrochloride.

During the period before irregularities appeared, no significant change in pulmonary pressure could be observed with doses ranging between 0.1 mgm. and 1.0 mgm. Coronary sinus outflow decreased approximately 10 per cent in experiment 21 after 0.2 mgm.

II. EFFECT UPON HEART RATE AND ELECTROCARDIOGRAM. Toxic doses of erythrophleum alkaloids caused one or more of the following effects: (1) a slowing of the heart; (2) an impairment of the conduction system manifested by a prolonged P-R interval and auricular-ventricular block; and (3) an increased spontaneity of the ventricles, as evidenced by ectopic beats and ventricular tachycardia. These effects upon heart rate and electrocardiogram first became obvious in from 10 to 25 minutes after an initial dose of an erythrophleum alkaloid. The onset of irregularities, however, was sometimes as brief as two minutes after a second or third dose of the alkaloid. The irregularities were usually long-lasting, continuing until the death of the heart or the cessation of the experiment an hour or more after the onset of the irregularities.

40 minutes following the first distinct sign of ventricular irregularities; the auricular rate, however, decreased gradually during this period from 157 to 120/min. During the first 30 minutes of this same period, the ventricles were beating irregularly at a rate approximately the same as that of the auricles. There were brief periods during which the ventricles were beating about 15 per cent faster than the auricles. The ventricular rate decreased considerably, however, and became regular between 30 and 39 minutes after the initial appearance of irregularities. Sixty-nine minutes after the dose of cassaine (39 minutes after the first appearance of irregularities), the ventricular rate was 44, while the auricular rate was 132, a three-to-one block; the ventricular complexes were regular and separated by a prolonged P-R interval from the auricular beat. This three-to-one block continued for about four minutes while the auricular rate decreased further to 120/min. The auricles then stopped beating. The ventricles gave four irregularly timed additional beats and also stopped beating.

C. *Spontaneity of the ventricles.* An increased spontaneity of the ventricles, manifested by ectopic beats and ventricular tachycardia, was the most frequently observed toxic effect of large doses of erythrophleum alkaloids. Transient outbursts of ventricular extrasystoles were observed in experiment 7 with cassaine and in experiment 13 with nor-cassaidine. Complete auricular-ventricular dissociation and ventricular tachycardia occured in experiment 3 with cassaine, in experiments 10 and 12 with nor-cassaidine, in experiments 14, 18, and 19 with erythrophleine, and in experiments 21, 22, and 23 with coumingine. In experiments 22 and 23 ventricular fibrillation resulted from doses of 1.0 mgm. of coumingine hydrochloride. We have not observed ventricular fibrillation with any of the other erythrophleum alkaloids, probably because the doses we have employed were not sufficiently large.

Figure 4 shows the ventricular tachycardia produced by an injection of 0.25 mgm. of coumingine hydrochloride in experiment 23. The changes in heart rate and electrocardiogram in this experiment may be considered typical of all the experiments in which ventricular tachycardia occurred. In some experiments, however, the transition between a normal heart rate and the ventricular tachycardia was less abrupt.

In the experiment of figure 4 the only chronotropic effect which occurred within the first 15.5 minutes following the injection was an increase in rate from 118 to 120/min. The ventricular rate increased sharply from 120.5/min. to 220/min. between the 17th and 20th minutes. The auricles continued to beat throughout the rest of the experiment at a much slower rate (between 120/min. and 132/min.) than the ventricles.

The electrocardiographic changes which took place in the heart-lung preparation of figure 4 are shown in figure 5. The earliest change consisted of a decrease in the T wave (upper tracing, figure 5 B), which occurred gradually and was noticeable within about 7 minutes. By the time 16 minutes had elapsed, alternate ventricular complexes were markedly changed in character (fig. 5 C), suggesting the regular discharge of an ectopic ventricular pacemaker in addition leine alkaloid upon the conduction system was observed in only one experiment (No. 7). The first distinct change in the electrocardiogram consisted of

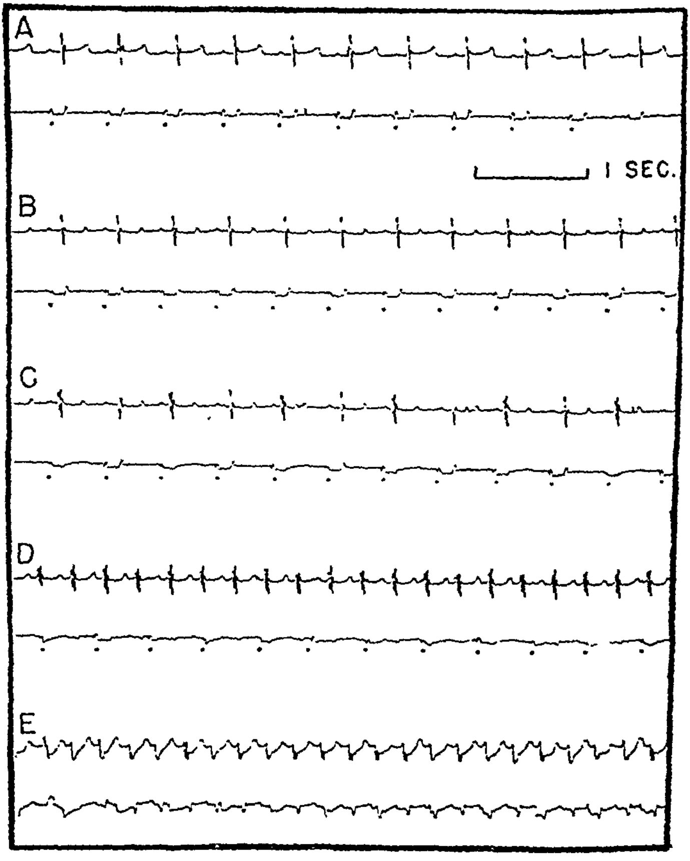

FIG. 5. EXPERIMENT NO. 23. ELECTROCARDIOGRAPHIC TRACINGS TAKEN FROM THE SAME EXPERIMENT AS FIGURE 4

The letters A, B, C, D, and E refer to the corresponding letters on figure 4. The upper tracing in each record was obtained with one electrode on the left side of the diaphragm and the other electrode on the left side of the lower neck. This tracing corresponds roughly to the standard lead III. The lower tracing in each record was obtained with one electrode in the rubber tubing connecting the right auricle to the venous manometer and the other electrode within the rubber tubing connected to the pulmonary artery. This tracing emphasizes the auricular contraction. Tracing A was recorded about 0.5 minutes before the injection of 0.25 mgm. coumingine hydrochloride. Tracings B, C, D, and E were recorded 15.5, 16, 18.5, and 26 minutes respectively after the injection. The horizontal rows of dots indicate the auricular contractions. It is difficult to analyze tracing E for auricular contractions even though observation of the heart at this time revealed a regular auricular rhythm of about 132/min.

ventricular premature beats coupled with normal beats. The total ventricular rate remained unchanged. The auricles continued to beat regularly for and rhythm of the heart. The changes in the rate of impulse production, the slowing of the rate of conduction of impulses, the increased spontaneity leading to irregularities of rate, ventricular tachycardia, and ventricular fibrillation are characteristic of all three groups of substances. Within each group the ratio between the minimal irregularity dose (m.i.d.) and the minimal positive inotropic dose (m.p.i.d.) appears to be characteristic of the individual compounds.

The difference between the lanata glycosides A, B, and C (although not apparent in the experiments of Cattell and Gold (28) on the isolated papillary muscle of the cat heart) is emphasized by Moe and Visscher (29) working on the heart-lung preparation of the dog. Similarly a marked difference was found between *veratridine and protoveratrine* (26). While our experiments with the erythrophleum alkaloids are not numerous enough to establish definite ratios of m.i.d. to m.p.i.d., our data indicate that nor-cassaidine and cassaine have a larger ratio than coumingine (see table 2).

TABLE 2

SUBSTANCE	MINIMAL POSITIVE INOTROPIC DOSE		MINIMAL IRREGULARITY DOSE		
	I	Mgm. per liter of blood	II	Mgm. per liter of blood	Ratio II/I
	mgm.		*mgm*		
Cassaine Hydrochloride	0.25	0.48	0.80	1.0	3
Nor-cassaidine Hydrochloride	<0.15	0.39	0.50	0.86	>3
Erythrophleine Sulfate	0.30	0.54	>0.50*	0.77*	>1.6
Coumingine Hydrochloride	0.10	0.16	0.10	0.16	1

* Irregularities occurred immediately after removal of Morawitz cannula.

As the only chemical difference between cassaine and coumingine is the esterification of the nuclear hydroxyl group with β-hydroxy *iso*valeric acid, this must therefore be connected with the increase in the toxic action of coumingine above that of cassaine. The difference in toxicity is obvious also from the data of Chen *et al.* (23), who found coumingine hydrochloride to be lethal for the cat at 0.15 mgm. per kgm., while a dose of 1.15 mgm. per kgm. of cassaine hydrochloide was required to kill the animal by heart action under the conditions accepted for the standardization of cardiac glycosides. Dalma (6) suggested that cassaine might prove useful clinically for its cardiac action. The choice of this alkaloid would be justified on the basis of the experimental evidence concerning its "therapeutic range"; however, no evidence is available as yet to justify the assumption that clinically in congestive heart failure the action of the erythrophleum alkaloids is truly digitalis-like.

SUMMARY

The cardiac action of the erythrophleum alkaloids, cassaine hydrochloride, nor-cassaidine hydrochloride, erythrophleine sulfate, and coumingine hydrochloride, was studied in the heart-lung preparation of the dog. All the

to the normal sinus node pacemaker. A tracing taken about 40 seconds after figure 5 C reveals three distinct types of ventricular complexes, which may have resulted from the regular discharge of two ectopic pacemakers plus the normal pacemaker. The P-R intervals were essentially unchanged for the normal ventricular complexes (fig. 5 C), indicating the normal functioning of the auricular-ventricular conduction system. In record D of figure 5, which was taken 18.5 minutes after the injection of coumingine hydrochloride, the ventricular rate had increased to 208/min. The abnormal appearance of the ventricular complexes and their independence of the auricular contractions denote a ventricular tachycardia.

III. ERYTHROPHLEIC ACID. The action of erythrophleic acid upon the mammalian heart was observed in one heart-lung preparation. A dose of 5 mgm. erythrophleic acid (equivalent to 5.5 mgm./100 grams of ventricle or 6.4 mgm./liter of blood) did not produce any changes in the performance or the electrocardiogram of the normal heart. Heart failure was then induced by the administration of sodium pentobarbital. No significant improvement (decrease in venous pressure and increase in systemic output) of the failing heart and no change in rate or alterations in the electrocardiogram resulted from a total of 37 mgm. erythrophleic acid (equivalent to 41 mgm./100 grams of ventricle or 61 mgm./liter of blood), administered over a period of 9 minutes.

DISCUSSION. Like the cardiac glycosides and the veratrum alkaloids, the erythrophleum alkaloids have a positive inotropic action, comparable insofar as the onset, extent and duration of the decrease in ventricular volume, and of the improvement of the work capacity in the failing heart are similar and are produced by doses of same order. As no metabolic studies have been carried out with the erythrophleum alkaloids (or the veratrum alkaloids) it is not possible to say whether the similarity to the action of the cardiac glycosides also holds for the changes in efficiency so characteristic for this group of pharmacological agents.

A comparison of the three groups of substances makes it clear that the effect studied by us in the isolated mammalian heart does not depend upon compounds of the same or closely related chemical structure. While the veratrum alkaloids have in common with the cardiac glycosides the sterol nature and differ from them in that a heterocyclic ring system takes the place of the unsaturated lactone attached to C_{17}, the erythrophleum alkaloids are neither sterols nor unsaturated lactones. The ester nature appears to be indispensable for the effect of the erythrophleum alkaloids, as erythrophleic acid itself in a dose more than 100 times as large as the minimal positive inotropic amount of erythrophleine proved ineffective. However, no information is available on the action of mono-, or dimethylaminoethanol in the normal or failing heart (27). Furthermore it is to be remembered in this connection that the veratrum alkamine cevine retains the positive inotropic action although more than 100 times the dose of the esteralkaloid veratridine is required to obtain a comparable effect.

The similarity between the erythrophleum alkaloids and the cardiac glycosides and veratrum alkaloids also holds with regard to the changes in rate

INFLUENCE OF EPINEPHRINE AND ATROPINE ON THE PULMONARY PRESSOR ACTION OF HISTAMINE[1]

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It is well established that atropine and epinephrine counteract some but not all of the effects of histamine. However, the few investigations which concern the antagonistic relationship on the pulmonary circulation were performed upon isolated pulmonary vessels: atropine was reported to remove the vasoconstrictor action of histamine (1, 2) and epinephrine was found to have no effect (2). Yet, in another area (coronary vessels) where histamine also causes vasoconstriction, epinephrine does cause vasodilation (3). Since the above studies were performed on isolated pulmonary vessels, it seemed advisable to investigate the antagonistic relationships in animals with intact circulatory systems.

Methods. Cats were selected as the test animal since histamine produces pulmonary constriction in most of them (4, 5). The animals were narcotized with pentobarbital-sodium. The chest was opened and the pulmonary vessels exposed using a technique similar to that described by Johnson, Hamilton, Katz and Weinstein (6). Cannulae connected to Hamilton manometers were inserted into a branch of the pulmonary artery, into a branch of the pulmonary vein, and into the carotid artery. Simultaneous recordings of the pulmonary arterial and pulmonary venous pressures allowed passive rises to be differentiated from active rises. A passive rise from back pressure from the left ventricle would elevate both the pulmonary venous and the pulmonary arterial pressure. On the other hand any increased resistance in the lung vessels would elevate the pulmonary arterial pressure and have no effect or slightly reduce the pulmonary venous pressure.

Results. In cats as previously reported (4) intravenous injections of histamine lowered the systemic and increased the pulmonary arterial pressure, see fig. 1. As previously reported from this laboratory (5) histamine increases the pulmonary peripheral resistance as shown 1) by the larger pressure gradient between the pulmonary artery and vein and 2) by the slower rate of pressure descent during diastole at any given pressure. The large pulmonary pulse pressure also indicates that the pulmonary arterial "Windkessel" is reduced in size by histamine. An increase in the pulmonary peripheral resistance and a reduction in the pulmonary "Windkessel" indicates constriction of both the pulmonary arteries and arterioles and possibly the venules.

In one of three animals histamine slowed the heart from 192 to 120 beats per minute, see fig. 1. Atropine abolished this cardiac slowing. At first glance it might appear that the increase in systemic pulse pressure was responsible for the cardiac slowing. However, this is not the case since the slowing persisted even after the systemic pulse pressure returned to the pre-injection amplitude. It apparently did not result from inadequate coronary flow since evidence of cardiac

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[1] Aided by a grant from Eli Lilly and Company.

alkaloids have a positive inotropic action which leads to an increase of the work of the heart, particularly in experimental heart failure. In large enough doses all the alkaloids lead to changes in heart rate and to irregularities of rhythm Our data indicate that nor-cassaidine and cassaine have a larger ratio between the minimal irregularity dose and the minimal positive inotropic dose than coumingine

Erythrophleic acid is devoid of any characteristic action upon the heart in a dose more than 100 times larger than the minimal positive inotropic dose of erythrophleine sulfate

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Large parasympatholytic doses of atropine did not counteract either the systemic depressor or the pulmonary pressor actions of histamine, see fig. 2. However, after atropine, the pulmonary pressure rise from histamine developed somewhat more slowly. This conceivably could originate from partial interference with the constricting action of histamine upon the arteries while the arterioles were unaffected by the atropine.

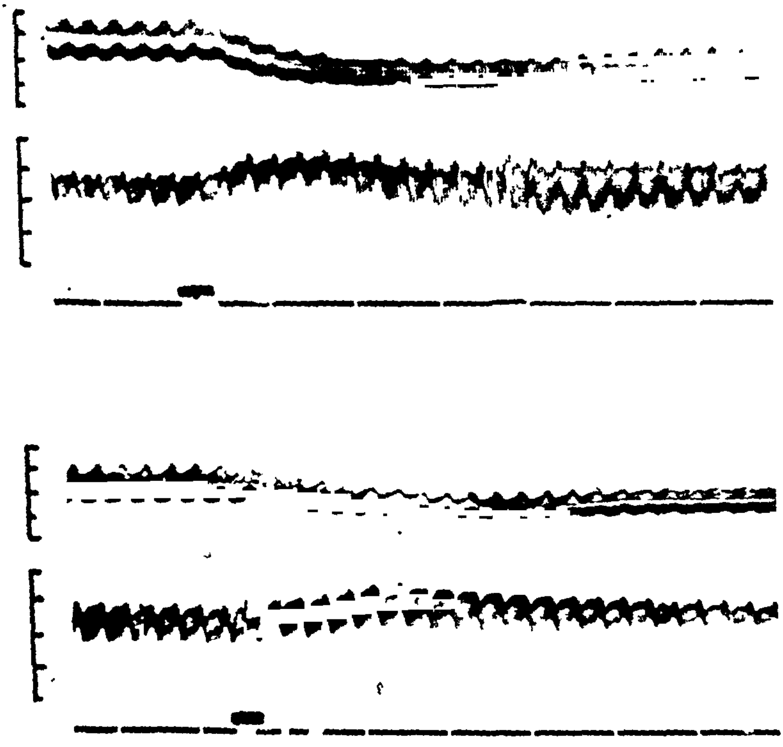

Fig. 2. Pressure pulses from cat which had received 30 mgm./kgm. of pentobarbital-sodium intraperitoneally. Tracings similar to those of fig. 1 except pulmonary venous records were omitted. At both signals 0.25 mgm./kgm. of histamine phosphate was injected intravenously. Twenty minutes elapsed between the upper and lower records. Five minutes prior to start of lower record 2.5 mgm./kgm. of atropine sulfate was injected intravenously. Note that the previous injection of atropine failed to significantly influence the pressure effects of histamine on either the systemic or pulmonary circulatory systems.

The failure of atropine to counteract the pulmonary effects of histamine disagrees with the observations on isolated pulmonary arteries. Possible explanations which can be offered concern (1) the difference in dosages of, (2) the tissues involved, and (3) the differences in species. In this study the intravenous injection was 2.5 mgm./kgm. while in the isolated artery the concentration was 1:5000, which roughly would correspond to 200 mgm./kgm.

hypoxia such as irregularities and weakness were absent. The slowing could have originated by vagal reflex from the elevated pressures in the right heart and in the pulmonary artery. However, this is unlikely, since right ventricular pressures as great as 100 mm. of mercury have been observed in humans without excessive cardiac slowing (7). Evidently histamine slowed the heart through the cholinergic mechanism but it is possible that the large pulmonary pulse pressure of 50 mm. of mercury contributed to the slowing.

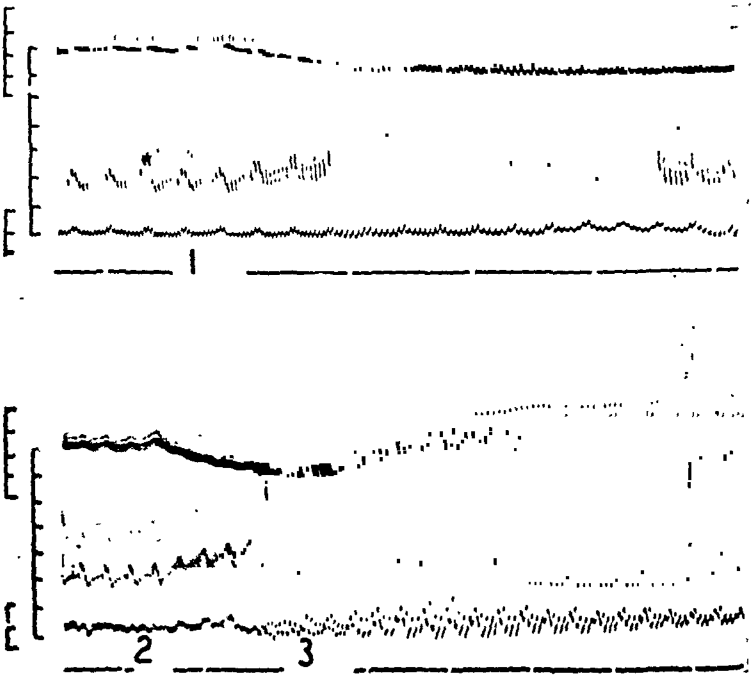

FIG. 1. From above downwards, pressure pulses from the carotid, pulmonary artery and pulmonary vein of a cat which had received 25 mgm./kgm. of pentobarbital-sodium intraperitoneally. Animal received artificial respiration. Time intervals of ten seconds are shown on the base line. Pressure scale of carotid shows units of 50 mm. Hg, while those of pulmonary artery and vein show units of 10 mm. Hg. At first signal 0.25 mgm./kgm. of histamine phosphate was injected into the femoral vein. Note the fall in systemic pressure and the rise in pulmonary arterial pressure and pulse pressure. The increase in the pressure gradient between the pulmonary artery and the pulmonary vein proves that the peripheral resistance of the pulmonary circulation had been increased by histamine. Forty minutes elapsed between the upper and the lower record. At the second signal the same dose of histamine was repeated and at signal three 0.05 mgm./kgm. of epinephrine was injected into the femoral vein. This elevated the systemic pressures to values above normal but failed to have any significant effect on the elevated pulmonary pressures.

Epinephrine completely eliminated the systemic depressor effects of histamine but failed to eliminate the pressor action of histamine on the pulmonary circulation, see fig. 1 signal 3.

CERTAIN INHIBITORY PROPERTIES POSSESSED BY A HOMOLOGOUS SERIES OF *p-n*-ALKYLOXYPHENYLETHYLAMINES

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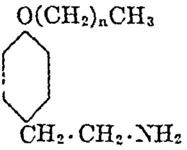
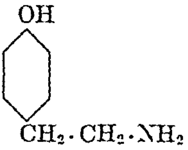
During the course of an investigation of the pharmacology of a series of *p-n*-alkyloxyphenylethylamines, it was noted that certain members of this series either greatly depressed or completely abolished the normal inhibitory action of the vagus on the heart. They had also an inhibitory action on isolated smooth muscle.

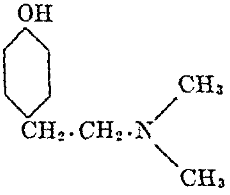
Some of these properties might have been anticipated from their structural relationships to other substances possessing similar properties.


$O(CH_2)_nCH_3$
$CH_2 \cdot CH_2 \cdot NH_2$

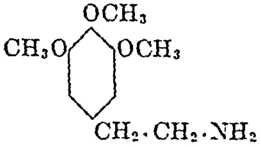
p-n-alkyloxyphenylethylamines


OH
$CH_2 \cdot CH_2 \cdot NH_2$

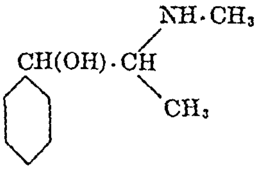
Tyramine


OH
CH_3
$CH_2 \cdot CH_2 \cdot N$
CH_3

Hordenine


OCH_3
CH_3O
OCH_3
$CH_2 \cdot CH_2 \cdot NH_2$

Mescaline


$NH \cdot CH_3$
$CH(OH) \cdot CH$
CH_3

Ephedrine

Thus, as long ago as 1910 Dale, Laidlaw and Symons (1) showed that hordenine methiodide in common with tropine and nicotine possesses the property of depressing the action of the vagus on the heart. Mescaline, which is trimethoxyphenylethylamine, has been found by Raymond-Hamet (2) to act similarly. The latter author has also shown (3) that tyramine, which may be

This study on cats concerns the entire pulmonary system including the arterioles whereas the isolated organ studies dealt only with small pulmonary arteries isolated from the rest of the rabbit.

CONCLUSIONS

In cats, epinephrine in doses sufficient to overcome the systemic depressor effects of histamine has little or no influence on the elevated pulmonary pressures from histamine. Large parasympatholytic doses of atropine do not counteract the systemic depressor nor the pulmonary pressor effects of histamine.

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rat and excised intestine from the rabbit failed to respond to the action of any of the common stimulants. This phenomenon was observed in the case of the substances tabulated below:

CAT	RABBIT	RAT	GUINEA PIG
Pituitary extract	Adrenaline	Pituitary extract	Tyramine
Acetyl choline	Ergotoxine	Acetyl choline	Histamine
Barium chloride	Histamine	Barium chloride	Ergot
	Pituitary extract		Acetyl choline
	Tyramine		Pituitary extract
	Acetyl choline		

In all our experiments on isolated tissue we used an isolated organ bath similar in type to that described by Burn and Dale (11). The uterine tissue was suspended in Ringer solution made up according to the formula of the same authors (loc.cit.) whilst intestinal tissue was immersed in Tyrode solution.

The procedure followed in all cases was to obtain a submaximal contraction with the stimulant under examination and then, after changing the Ringer solution, to add a known amount of the alkyloxy derivative and to allow it to act upon the muscle for a definite period, usually 5 minutes. This period was selected because it was found to be suitable in the ergotoxine-adrenaline reversal experiments on the rabbit uterus described by Broom and Clark (12). Without changing the Ringer solution, the original concentration of stimulant was then added to the bath and the presence or absence of a response observed. Finally, the Ringer solution was changed and the dose of stimulant originally used was repeated. In the case of the lower members of the series, the magnitude of the response was now equal to that obtained before the alkyloxy derivative was added. With the higher members however, several changes of Ringer were sometimes necessary before the normal response was obtained. A typical result is shown in fig. 1.

The uteri of the guinea pig, cat and rabbit were all stimulated by the methoxy and ethoxy derivatives and it was impossible to study their inhibitory action on these tissues because they were still contracted at the time when the stimulant being studied should have been added to the bath. Contrary to the observations of Epstein, Gunn and Virden (20) who recorded that the methoxy compound stimulated smooth muscle directly in rodents we found that both the methoxy and ethoxy compounds failed to stimulate the uterus of the rat and this tissue was used to determine to what extent they were able to exercise the same inhibitory action as the higher members of the series.

pounds, however, failed to stimulate the uterus of the rat and this tissue was used to determine to what extent they were able to exercise the same inhibitory action as the higher members of the series.

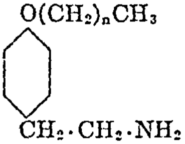
That they do to some extent possess this property is seen from fig. 2 which illustrates on the rat uterus, the partial inhibition of acetyl choline by *p-n*-ethoxyphenylethylamine.

regarded as the parent substance of all these compounds, depresses the excitability of the cardiac fibres of the vagus.

Curtis (4) has reported that the presence of ephedrine in an isolated organ bath containing a strip of intestine prevents a subsequent dose of adrenaline from producing its normal inhibitory effect. This observation has been confirmed by Finkleman (5) who has also shown that, in the presence of ephedrine, sympathetic stimulation fails to produce intestinal inhibition. Reintz (6) found that low concentrations of ephedrine increased the effect of adrenaline on the rabbit uterus, but high concentrations diminished or abolished it.

The *p*-*n*-alkyloxyphenylethylamines also reduce and sometimes abolish the pressor action of adrenaline, and, in this case also, a closely related series of compounds has been found to possess similar properties. Thus Levy and his co-workers (7, 8 and 9) described a number of phenoxyphenylethylamine derivatives of the types of $C_6H_5O \cdot CH_2 \cdot CH_2 \cdot NRR_1$ and $CH_3O \cdot C_6H_4 \cdot O \cdot CH_2 \cdot CH_2NRR_1$ and found that they suppress or reverse certain of the pharmacological effects of adrenaline. The actual results obtained varied with the animal used. They found for instance, that whilst many of these substances would reverse the hypertensive action of adrenaline in the dog, they failed to do so in the rabbit. Similar inhibitory properties have also been ascribed by Raymond-Hamet (10) to *p*-hydroxyphenylethyldimethylamine (Hordenine), phenylethyldimethylamine and phenylethyldiethylamine.

Experimental. The following eight members of the series of *p*-*n*-alkyloxyphenylethylamines have been examined.



p-methoxyphenylethylamine hydrochloride $n = 0$
p-ethoxyphenylethylamine hydrochloride $n = 1$
p-*n*-propoxyphenylethylamine lactate $n = 2$
p-*n*-butoxyphenylethylamine lactate $n = 3$
p-*n*-amyloxyphenylethylamine lactate $n = 4$
p-*n*-hexoxyphenylethylamine lactate $n = 5$
p-*n*-heptoxyphenylethylamine lactate $n = 6$
p-*n*-nonoxyphenylethylamine lactate $n = 8$

They are all derivatives of tyramine, in which substitution has been effected in the hydroxyl group and, in each case, the normal alkyloxy derivative has been prepared. The hydrochlorides of the two lowest members of the series were used but, on account of their increased solubility, the lactates of the higher members were found to be more suitable for pharmacological examination.

Inhibitory action on isolated tissue. Experiments on isolated uterine muscle revealed the fact that, in the presence of members of this series of *p*-*n*-alkyloxyphenylethylamines, excised uteri from the rabbit, guinea pig, cat and

Table 1 shows the concentrations of *p*-*n*-butoxyphenylethylamine which will inhibit the action of specified concentrations of various drugs. Table 2 gives the concentration of each member of the *p*-*n*-alkyloxyphenylethylamine series necessary to prevent the action on the isolated guinea pig uterus of tyramine in a concentration of 1:20,000.

TABLE 1

Minimum concentration of p-n-alkyloxyphenylethylamines necessary to desensitize the uterus to the action of smooth muscle stimulants

PREPARATION	CONCENTRATION OF *p*-*n*-BUTOXYPHENYLETHYLAMINE	ABOLISHES ACTION OF
Rat uterus	1:10,000	Pituitary ext. 1 unit:5000
	1:20,000	
Guinea pig uterus	1:10,000	Pituitary ext. 1 unit:3300
	1:12,000	Histamine 1:36M
	1:5,000	Ext. ergot. liq. B P. '14 1:500
	1:10,000	Acetyl choline 1:2M
	1:20,000	Tyramine 1:20,000
Rabbit uterus	1:10,000	Acetyl choline 1:1M
	1:5,000	Histamine 1:1M
	1:10,000	Tyramine 1:20,000
	1:10,000	Pituitary ext. 1 unit:500
	1:5,000	Adrenaline 1:1M
	1:2,500	Ergotoxine 1:2.6M

1. The concentrations of *p*-*n*-butoxyphenylethylamine given in the second column are not necessarily the lowest which will inhibit the concentration of the substances listed in the last column, but are the lowest concentrations used in our experiments which produced the effect recorded.

2 The concentrations given in the table represent the final concentrations in the isolated organ bath.

TABLE 2

Concentration of p-n-alkyloxyphenylethylamines necessary to inhibit tyramine 1:20,000 on the guinea pig uterus

DERIVATIVE	MINIMUM EFFECTIVE CONCENTRATION OF *p*-*n* ALKYLOXYPHENYLETHYLAMINES	INHIBITING EFFICIENCY FACTOR
Ethoxy	1:2,000	0.05
Propoxy	1:10,000	0.25
Butoxy	1:40,000	1
Amyloxy	1:80,000	2
Hexoxy	1:120,000	3
Heptoxy	1:100,000	2.5
Nonoxy	1:50,000	1.25

It is possible with the aid of tables 1 and 2, to calculate the approximate concentration of any member of the alkyloxy series which will with certainty inhibit

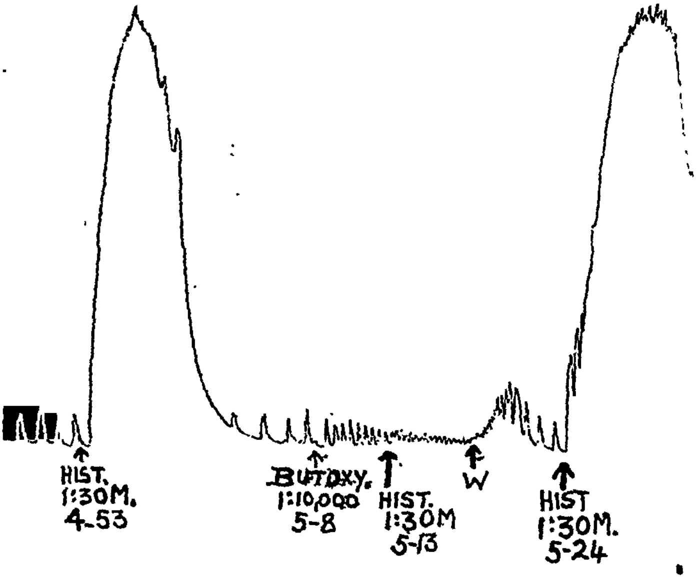


Fig. 1. Shows Inhibitory Effects of *p*-*n*-butoxyphenylethylamine on the Contraction of the Guinea-Pig's Uterus Induced by Histamine

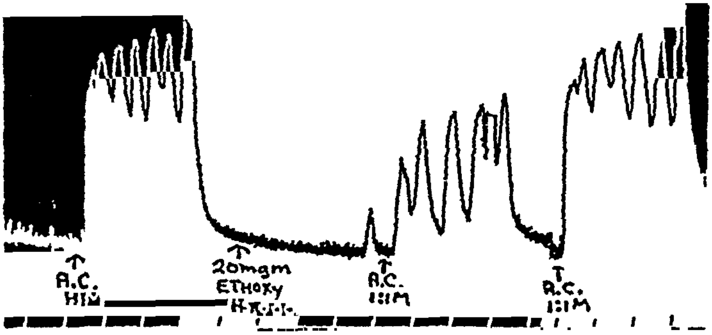


Fig. 2. Shows the Partial Inhibitory Effect of Ethoxyphenylethylamine on the Contraction of the Rat's Uterus Induced by Acetyl Choline

therefore chosen for these experiments. It was found that the addition of a solution of tyramine acid phosphate to bring the concentration of tyramine base in the isolated organ bath to 1:1000 abolished the action of acetyl choline 1:2M, pituitary extract 1 international unit in 2,500 and almost completely abolished the effect of barium chloride 1:5000 (fig. 5). On the other hand, tyramine in this same concentration did not influence the inhibitory effect of adrenaline 1:5M (fig. 6).

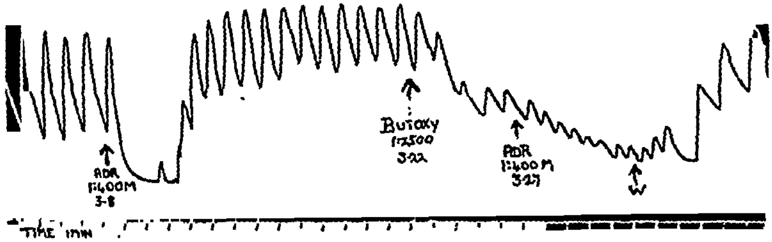


Fig. 4. Shows the Lack of Inhibitory Effect of Butoxyphenylethylamine on the Relaxation of the Rat's Uterus Induced by Adrenaline
The same concentrations were used as in fig 3

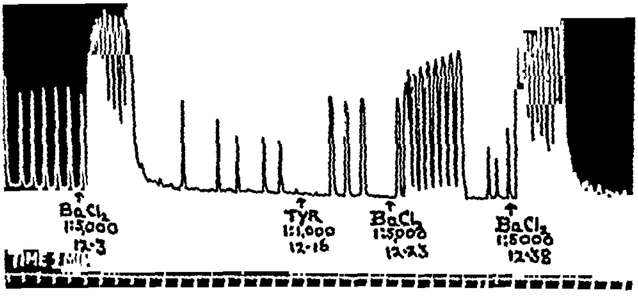


Fig. 5. Shows the Inhibitory Effect of Tyramine on the Contraction of the Rat's Uterus Induced by Barium Chloride

It would seem therefore, that, provided a sufficiently high concentration is used, tyramine as well as its alkyl ethers, can render isolated tissue insensitive to the action of all substances giving motor effects, although in the concentrations used in our experiments, it is unable to inhibit the action of adrenaline on tissues which respond by relaxation.

the effect of a given concentration of any of the substances listed in column 3 of table 1. This is determined by multiplying the concentration of the butoxy derivative (from table 1) which will produce the desired effect, by the inhibitory efficiency factor (from table 2) of the alkyloxy derivative which it is proposed to use. For example, to inhibit the action of histamine 1:1M on the rabbit uterus with *p-n*-heptoxyphenylethylamine it would be necessary to use a concentration of 1:5,000 × 2.5 = 1:12,500.

The inhibitory effects just described were of a very transient nature, being much less prolonged than the effect of ergotoxine on the rabbit uterus. In most cases a normal response to drugs was obtained in less than 5 minutes after the Ringer solution had been changed. There was, however, more delay when the higher members of the series were used. This is probably because the higher members are precipitated in serum and saline. Precipitation also occurs in the isolated organ bath, and hence it is more difficult to wash the suspended tissue free from precipitated particles of the higher members, than to remove the lower members which are completely soluble in Ringer solution.

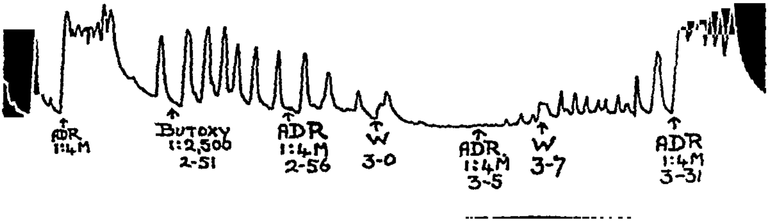


FIG. 3. SHOWS THE INHIBITORY EFFECT OF BUTOXYPHENYLETHYLAMINE ON THE CONTRACTION OF THE RABBIT'S UTERUS INDUCED BY ADRENALINE

RELATION OF THE *p-n*-ALKYLOXYPHENYLETHYLAMINES TO THE ACTION OF ADRENALINE ON THE UTERUS. Adrenaline contracts the rabbit uterus, but produces relaxation in the uterus of the non-pregnant cat, guinea pig and rat. Our experiments showed that members of the *p-n*-alkyloxyphenylethylamine series would inhibit the effect of adrenaline on the rabbit uterus which responds by contraction but had no such effect on the uteri of cat, guinea pig or rat, which are inhibited by adrenaline. This is seen from fig. 3, which shows the complete suppression of the action of adrenaline 1:4M on the rabbit uterus by *p-n*-butoxyphenylethylamine in a concentration of 1:2,500 and fig. 4 in which the same dose of the butoxy derivative fails to abolish the effect of adrenaline 1:400M on the rat uterus.

INHIBITORY PROPERTIES OF TYRAMINE. In view of the chemical relationship between tyramine and the *p-n*-alkyloxyphenylethylamines, it appeared of interest to study its inhibitory properties in a similar manner. Tyramine contracts practically all isolated tissues except that of the rat uterus, which was therefore chosen for these experiments. It was found that the addition of a solution of tyramine acid phosphate to bring the concentration of tyramine base in the isolated organ bath to 1:1000 abolished the action of acetyl choline 1:2M, pituitary extract 1 international unit in 2,500 and almost completely abolished the effect of barium chloride 1:5000 (fig. 5). On the other hand, tyramine in

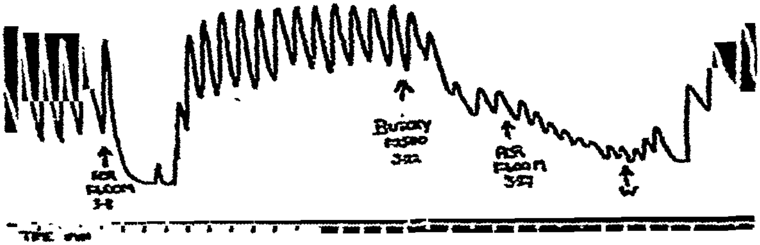


FIG. 4. SHOWS THE LACK OF INHIBITORY EFFECT OF BUTOXYPHENYLETHYLAMINE ON THE RELAXATION OF THE RAT'S UTERUS INDUCED BY ADRENALINE
The same concentrations were used as in fig. 3

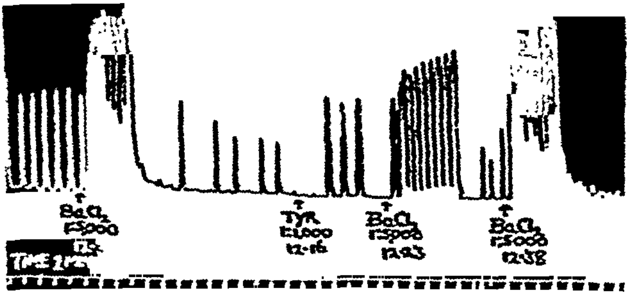


FIG. 5. SHOWS THE INHIBITORY EFFECT OF TYRAMINE ON THE CONTRACTION OF THE RAT'S UTERUS INDUCED BY BARIUM CHLORIDE

this same concentration did not influence the inhibitory effect of adrenaline 1:5M (fig. 6).

It would seem therefore, that, provided a sufficiently high concentration is used, tyramine as well as its alkyl ethers, can render isolated tissue insensitive to the action of all substances giving motor effects, although in the concentrations used in our experiments, it is unable to inhibit the action of adrenaline on tissues which respond by relaxation.

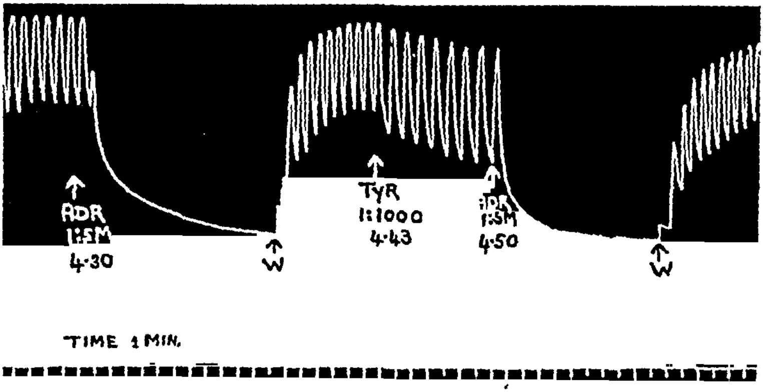


FIG. 6. SHOWS THE LACK OF INHIBITORY EFFECT OF TYRAMINE ON THE RELAXATION OF THE RAT'S UTERUS INDUCED BY ADRENALINE

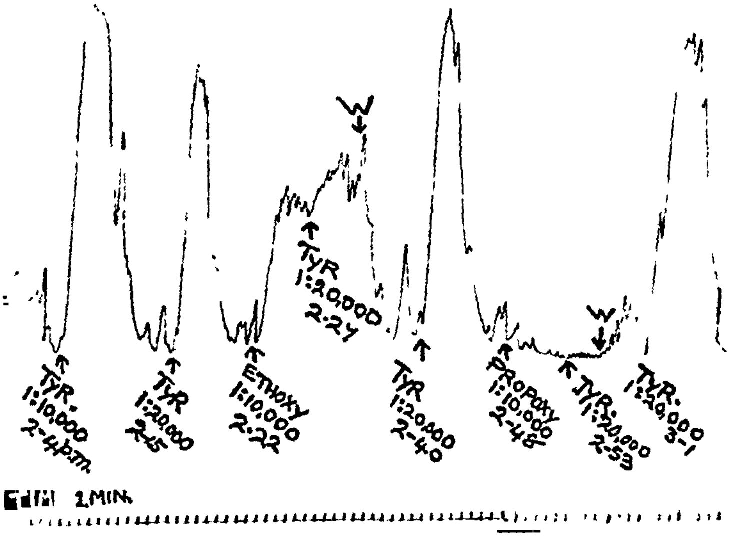


FIG. 7. SHOWS THAT THE CONTRACTION OF THE GUINEA PIG'S UTERUS INDUCED BY TYRAMINE IS COMPLETELY INHIBITED BY PROPOXYPHENYLETHYLAMINE BUT NOT BY THE SAME CONCENTRATION OF THE ETHOXY DERIVATIVE

EFFECT OF CHEMICAL STRUCTURE ON INHIBITORY ACTIVITY. The gradual change in activity as one proceeds from member to member of this series, is well illustrated by the different inhibitory activities of the ethoxy and propoxy derivatives against tyramine on the guinea pig uterus. Fig. 7 shows that, whilst the ethoxy derivative in a concentration of 1:10,000 fails to inhibit the action of tyramine 1:20,000, the same concentration of the propoxy derivative produces complete inhibition. Table 2 expresses the same effect quantitatively and shows the minimum concentrations of the individual members necessary to inhibit the stimulant action of tyramine 1:20,000. It will be seen that the activity increases with increase in the length of the alkyloxy side chain, until peak activity is reached with the hexoxy derivative, after which a decline in activity occurs. The methoxy derivative is not included in this series because this type of experiment cannot be carried out with this member on the guinea pig uterus owing to its great and prolonged stimulant action.

MECHANISM OF INHIBITORY ACTION ON ISOLATED TISSUE. It appeared that the property possessed by tyramine and the *p-n*-alkyloxyphenylethylamines of suppressing the action of common stimulants might be due to any one of three causes.

1. An associated anaesthetic property of the inhibitant.
2. Chemical reaction between the inhibitant and stimulant.
3. The property of the inhibitant of blocking the approach of the stimulant to the effector muscle cells.

The possibility that the inhibitory properties just described might be associated with a general anaesthetic action of the alkyloxy derivative was suggested by the work of Lieb and Mulinos (13) confirmed by Garry (14) who showed that the anaesthetic amytal causes a prolonged but temporary inhibition of vagal action on the heart and reduces the motility of the intestine. Similar results were obtained by Hauschild and Lendle (15) who showed that the adrenaline reversal by ergotoxine is abolished by narcotics which they suggest alter the response of the end organ.

Experiments in which doses up to 100 mgm./kgm. of the butoxy compound were injected intraperitoneally into rats, showed that this particular member of the alkyloxy series did not possess any general anaesthetic properties. Similarly, experiments in which the same compound was instilled into the rabbits cornea failed to indicate any local anaesthetic activity.

The possibility that the inhibitory effect of these substances might be caused by the formation of an inactive chemical compound between the alkyloxy derivative and the stimulant was next considered. This, however, seemed improbable on chemical grounds since it would be very unlikely that the alkyloxy compounds would combine with such diverse substances as acetyl choline, the constituents of ergot, adrenaline and barium chloride.

The most likely explanation therefore seemed to be that the *p-n*-alkyloxyphenylethylamines attach themselves to some portion of the cell structure responsible for contraction thereby blocking the approach of a subsequently added stimulant.

Our experiments also showed that, whilst these substances inhibited the action of drugs which produce a contraction of smooth muscle, they fail to inhibit the action of drugs (e.g. adrenaline) which produce relaxation of smooth muscle.

It appeared of interest to investigate this point further and see if, irrespective of the type of stimulant substance used, the same concentration of one of the alkyloxy derivatives would inhibit a certain stimulant effect (as indicated by the height of the contraction) on a muscle such as the isolated uterus.

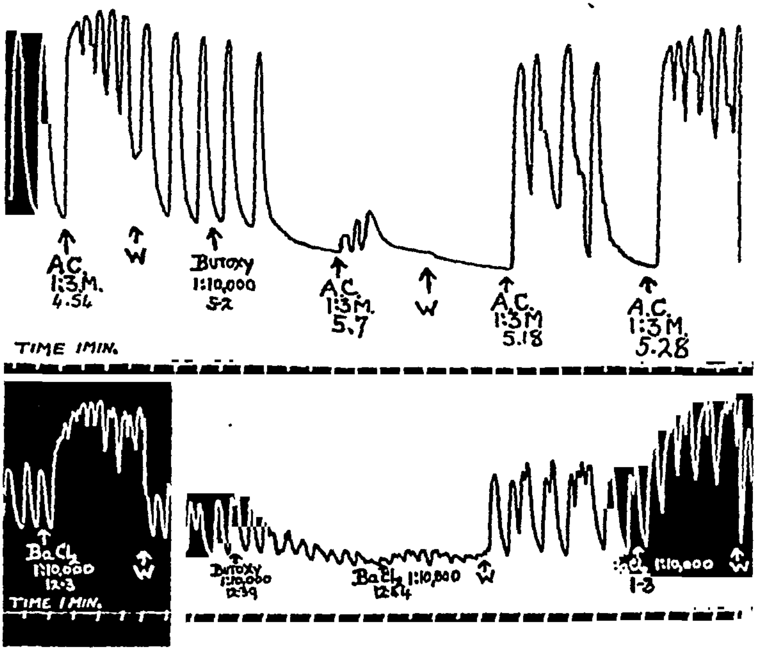


FIG. 8. SHOWS THAT EQUICONTRACTILE DOSES OF ACETYL CHOLINE AND BARIUM CHLORIDE ARE INHIBITED BY THE SAME CONCENTRATION OF BUTOXYPHENYLETHYLAMINE

The minimum effective concentrations of acetyl choline and barium chloride on a particular strip of rat uterus were determined. These were found to be acetyl choline $1:3 \times 10^6$ and barium chloride 1:10,000. The minimum concentrations of *p*-*n*-butoxyphenylethylamine which would suppress the effect of these concentrations of acetyl choline and barium chloride were then determined. These were found to be the same viz: 1:10,000. These results are shown in fig. 8.

On this same strip of rats uterus it was observed that the minimum effective concentration of adrenaline was 1:400M and that the relaxation produced by this concentration was not suppressed by the butoxy derivative in a concentration of 1:2,500 i.e. 4 times the concentration necessary to suppress the action of the minimum effective concentrations of acetyl choline and barium chloride (fig. 4).

It appears therefore that the concentration of alkyloxy derivative necessary to inhibit a given motor effect is dependent on the magnitude of the effect not on the substance producing it, and further, that it is not possible to influence an inhibitor effect in the same way even when much greater concentrations are used.

INHIBITORY EFFECTS ON THE INTACT ANIMAL. In view of the results obtained with these compounds on isolated tissue, we were interested to determine whether they possessed similar inhibitory properties when injected intravenously into the intact animal.

Uterus in situ. In these experiments both cats and rabbits were used. The animals were first anaesthetized, cats with chloralose (5 cc./kgm. saturated solution at 37°C. intravenously) and rabbits with dial (0.7 cc./kgm. 10 p.c. solution intraperitoneally). The anaesthetized animal was tied down to a special board, tracheotomy was performed and jugular and carotid cannulas inserted. The board and animal were then placed in an electrically heated bath containing physiological saline thermostatically controlled at 39°C. The abdomen was opened and the two levers of a Cushny myocardiograph were attached to a short length of uterus. The free lever was then attached by a thread to a writing lever, the carotid cannula attached to the manometer and the jugular cannula to the injection burette. The animal was then ready for experiment.

In none of our experiments did we observe any inhibitory effects on the cat or rabbit uterus in situ similar to those obtained on the isolated organ.

We were also unable to cause relaxation of a uterus already contracted by an injection of pituitary extract even when as much as 20 mgm. of the butoxy derivative was administered.

INFLUENCE ON PRESSOR ACTION OF ADRENALINE. It was noted in the case of the rabbit that the pressor response to a small dose of adrenaline injected 5–30 minutes after a large dose of the higher members of the alkyloxy series, was considerably greater than that produced by the same dose of adrenaline injected prior to the alkyloxy compound. This effect began about 5–10 minutes after the injection of the alkyloxy derivative and persisted for some 10–20 minutes, after which a normal response was obtained.

It had already been observed in experiments to be described later, that large doses of these compounds suppress the cardiac inhibitory action of the vagus and it was thought that the augmented pressor response to adrenaline after an alkyloxy derivative might be due to this action. It seemed possible that after their injection the vagal compensatory mechanism was unable to respond by cardiac slowing to a high blood pressure, and consequently the pressor response to adrenaline was increased. This, however, proved not to be the case for, even in animals in which both vagi were cut, the pressor response to adrenaline was increased by a previously injected large dose of an alkyloxy derivative. This is illustrated in fig. 9.

Although the augmentation of the response to adrenaline was frequently seen in the case of the rabbit it was never seen when the cat was the experimental animal.

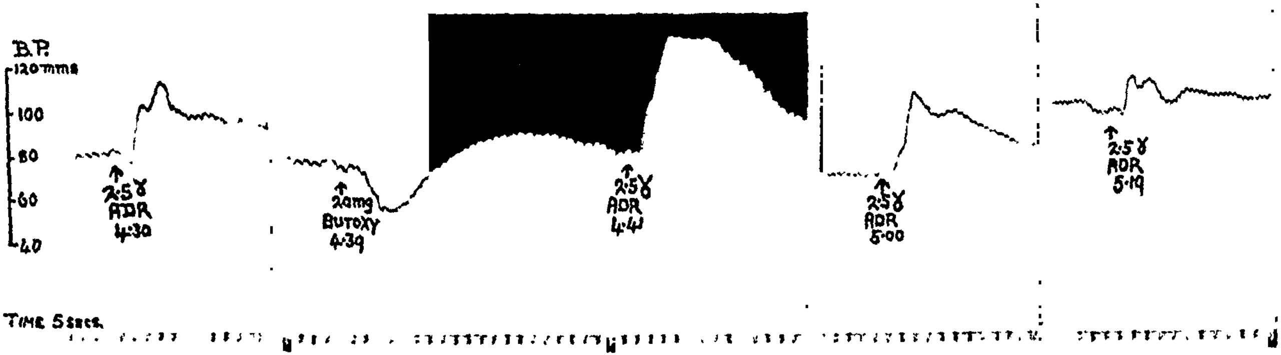


FIG. 9. RABBIT. SHOWS THE INCREASED PRESSOR RESPONSE TO ADRENALINE TWO MINUTES AFTER THE ADMINISTRATION OF BUTOXYPHENYLETHYLAMINE FOLLOWED BY A DIMINISHED RESPONSE FORTY MINUTES LATER

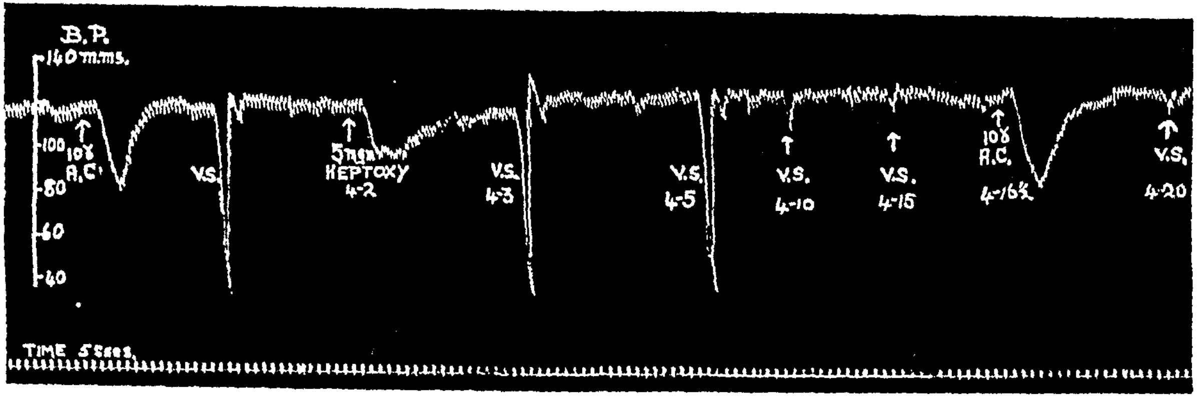


FIG. 10. RABBIT. SHOWS THE REDUCTION IN VAGAL RESPONSE AFTER HEPTOXY PHENYLETHYLAMINE UNACCOMPANIED BY ANY ALTERATION IN THE RESPONSE TO ACETYL CHOLINE

This period of increased sensitivity to adrenaline was of short duration and was in some cases succeeded by a period of reduced sensitivity. This point is also illustrated in fig. 9. The period of inhibition was noticed in both rabbits and cats although it did not occur in all our experiments.

ACTION ON THE VAGUS. With the exception of the methoxy compound, all the members of the series were found to inhibit the fall in blood pressure due to stimulation of the peripheral end of the vagus in both the rabbit and the cat. It was found that this inhibitory action on the vagus increased with increasing molecular weight. Thus whilst 20 mgm./kgm. of the ethoxy derivative only partially inhibited the effect of vagal stimulation, complete inhibition followed the intravenous injection of 1.9 mgm./kgm. of the heptoxy derivative.

INFLUENCE ON HYPOTENSIVE ACTION OF ACETYL CHOLINE. Since it is generally accepted that acetyl choline is released when cholinergic nerves are stimulated it was decided to investigate the action of these substances on the depressor effects of acetyl choline. As the *p-n*-alkyloxyphenylethylamines both suppress the effects of electrical stimulation of the cardiac fibres of the vagus and inhibit the effect of acetyl choline on isolated tissue, it was expected that they would also influence the hypotensive action of acetyl choline.

Experiment showed, however, that they had no marked effect on the depressor response to acetyl choline even in an animal in which they had completely desensitized the cardiac inhibitory fibres of the vagus. This is seen in fig. 10 which shows a typical reduction in the vagal response of the rabbit without a corresponding reduction in the hypotensive action of acetyl choline.

In this connection, it should be remembered that the circulatory changes produced by acetyl choline are due, in varying degree, to its action on the vessels themselves and on the heart, and that, of these, peripheral vasodilation is the principal cause of its depressor action. Whilst it appeared possible that these substances might be able to abolish the cardiac inhibitory action of acetyl choline, there was no evidence to suggest that they might affect its peripheral vascular action. This being so, one would expect the alkyloxy derivatives to affect only slightly the depressor action of acetyl choline because its vasodilator action would, almost certainly, tend to mask any inhibition of its cardiac action.

That the apparent anomalous behaviour of acetyl choline could be explained in this way was shown by the following experiment.

A cat was arranged for recording blood pressure and a Cushny myocardiograph was attached to the right ventricle of the heart. *p-n*-butoxyphenylethylamine was then injected into the jugular vein until the cardiac inhibitory action of the vagus was abolished. To do this one dose of about 20 and two doses of 10 mgm. were necessary. When the vagal action was completely abolished it was noted that acetyl choline failed to cause any change in either the rate or the amplitude of the heart beat, although it still produced a considerable, although slightly reduced, fall in blood pressure. When the full vagal response returned, acetyl choline produced the normal reduction in amplitude and slowing of the heart and a slightly greater fall in blood pressure.

The actual results obtained are shown in fig. 11, the last dose of *p-n*-butoxy-

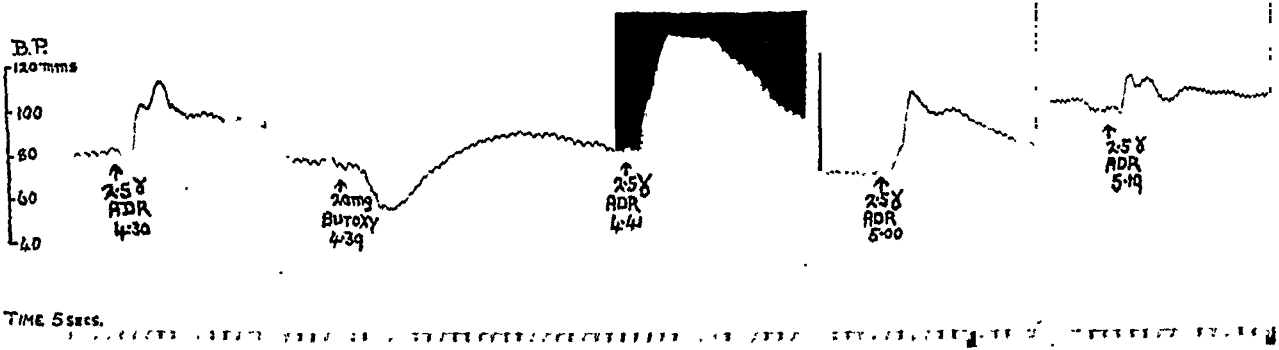


Fig 9. Rabbit. Shows the Increased Pressor Response to Adrenaline Two Minutes after the Administration of Butoxyphenylethylamine followed by a Diminished Response Forty Minutes Later

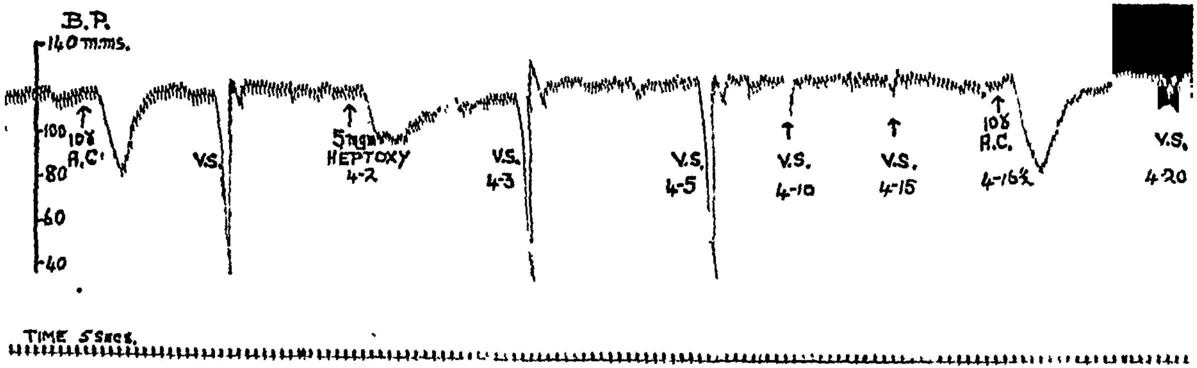


Fig. 10. Rabbit. Shows the Reduction in Vagal Response after Heptoxy phenylethylamine Unaccompanied by any Alteration in the Response to Acetyl Choline

phenylethylamine being injected 1½ minutes before the first vagus stimulation shown on the portion of the tracing reproduced.

In a subsequent experiment in which the change in leg volume was observed by means of a plethysmograph it was noted that, whereas acetyl choline had no cardiac action in a cat whose vagus had been paralyzed with *p-n*-butoxyphenylethylamine, the usual peripheral vasodilation took place.

DISCUSSION. The alkyloxyphenylamines were found to potentiate the pressor effect of adrenaline in the rabbit and sometimes this was followed by a period of reduced sensitivity to adrenaline. In this respect they resemble ephedrine. Gaddum (16) has suggested that ephedrine protects the adrenaline-like substance produced at adrenergic nerve endings by inhibiting the enzyme amine oxidase which normally destroys it. Larger doses of ephedrine may, however, block the muscle receptors and prevent adrenaline from acting. This suggestion arose out of the observations of Blaschko et al. (17) who showed that ephedrine is not destroyed by amine oxidase but in the presence of ephedrine the enzyme is prevented from destroying adrenaline. It is of interest, therefore, that Blaschko (personal communication) has shown that the alkyloxyphenylethylamines act as inhibitors of amine oxidase and it seems probable that they influence the pressor action of adrenaline through a mechanism similar to that of ephedrine.

The inhibitory effects of the alkyloxy compounds on isolated tissue cannot, however, be explained so simply. The same dose of one of these substances will inhibit the action of equi-contractile doses of all substances giving a motor effect, i.e., not only adrenaline but substances such as posterior pituitary extract and barium chloride. On the other hand, on those tissues in which adrenaline produces relaxation, this response is not affected by an alkyloxy derivative even in high concentrations. Clark (18) has pointed out that too sweeping deductions should not be made from a study of drug antagonisms. It seems probable, however, that all drugs act directly on the effector cells without the intervention of any intermediary mechanisms, but, to explain why the same substance may give in some tissues contraction and in others relaxation Loewi (19) suggests that differentiation may occur within the cell. This differentiation, which may be physiochemical rather than morphological, may conceivably take place in two directions, and the extent to which the cell is differentiated in one direction more than the other will determine whether a given tissue will respond to a particular drug by relaxation or contraction. The behavior of the alkyloxy derivatives in inhibiting the action of a substance on a tissue in which it causes contraction, and failing to do so on another tissue in which it produces relaxation can easily be explained on the basis of such a hypothesis. It must be assumed, however, that the alkyloxy derivatives attach themselves to that portion of the cell structure responsible for tissue contraction thereby blocking the approach of other substances which now fail to produce their normal response. Further it would appear that they do not attach themselves to that portion of the cell structure responsible for tissue relaxation, and consequently their presence in the cell does not influence the action of drugs producing relaxation.

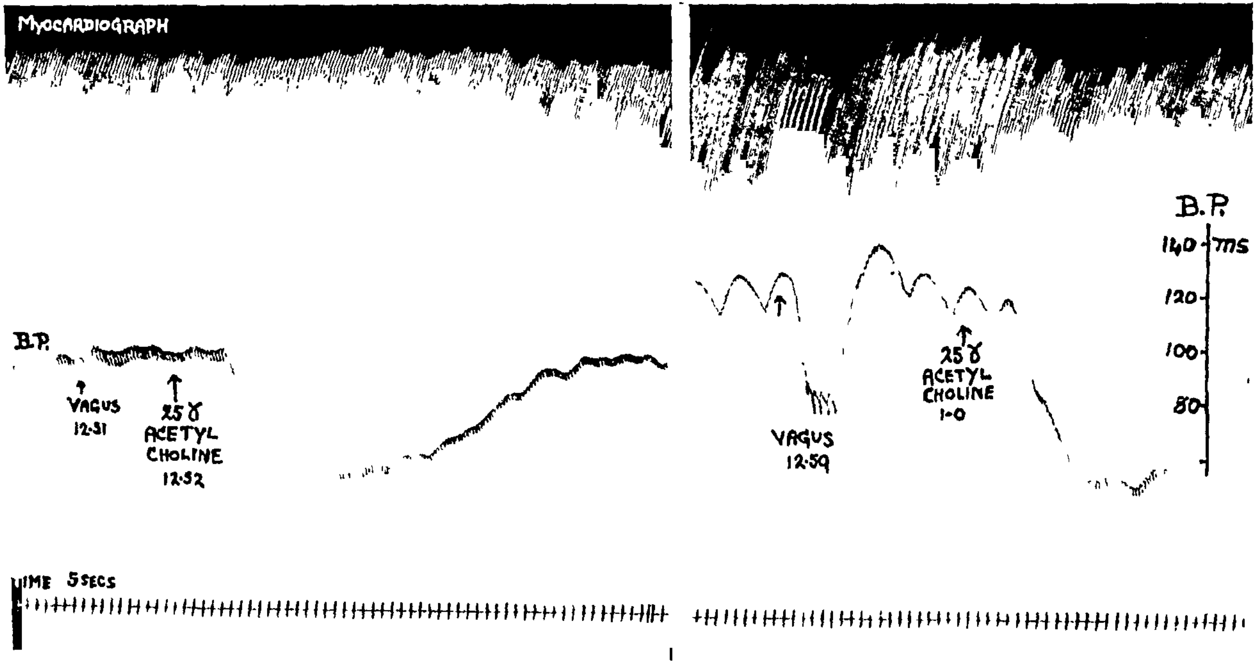


FIG. 11. CAT. VAGUS PARALYSED BY PREVIOUS DOSE OF BUTOXYPHENYLETHYLAMINE. SHOWS THAT THE DEPRESSOR RESPONSE TO ACETYL CHOLINE IS UNACCOMPANIED BY SLOWING OF THE HEART

When the action of the ethylamine derivative on the vagus has passed off the acetyl choline causes slowing of the heart and a greater fall of blood pressure.

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SUMMARY

1. Eight members of a homologous series of *p*-*n*-alkyloxyphenylethylamines have been examined and found to inhibit the action of certain drugs both on isolated smooth muscle and in the intact animal.

2. This inhibitory action has been studied on excised uteri from the rabbit, guinea pig, cat and rat and on the excised intestine from the rabbit.

It has been shown that the presence of a member of the *p*-*n*-alkyloxyphenylethylamine series in the isolated organ bath inhibits the action on smooth muscle of all the substances examined which produce a contraction of smooth muscle. The behaviour of these substances in relation to adrenaline is peculiar in that they inhibit its action on the uterus of the rabbit, where it causes the muscle to contract, but have no action on the uterus of the cat, guinea pig and rat, which are relaxed by adrenaline.

3. It is demonstrated that this inhibitory activity increases with increase in the length of the alkyloxy side chain until peak activity is reached with the hexoxy derivative after which a decline in activity occurs.

4. It is also shown that tyramine, as well as its alkyl ethers, can render isolated smooth muscle insensitive to the action of drugs which normally produce a contraction, but in the concentrations used in these experiments, tyramine fails to inhibit the action of adrenaline on tissues which respond by relaxation.

5. The inhibitory action of members of the alkyloxy series on smooth muscle is only observed on isolated tissue and their injection into the intact animal fails to influence the response of the uterus and intestine in situ to smooth muscle stimulants.

6. Members of the alkyloxy series inhibit the action of the vagus and acetyl choline on the heart, although they do not affect the peripheral vasodilatation caused by acetyl choline.

7. In the intact rabbit the members of the alkyloxy series sensitize the animal to the action of adrenaline. This effect soon passes off, and is sometimes followed by a period of reduced sensitivity. These substances do not sensitize the cat to adrenaline although the period of reduced sensitivity to adrenaline already referred to in the case of the rabbit, is also frequently seen with the cat.

8. The mechanism of the inhibitory action of the *p*-*n*-alkyloxyphenylethylamine on smooth muscle is discussed.

The authors wish to express their gratitude to Messrs. Boots Pure Drug Co. Ltd., who supplied the compounds used in this investigation.

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THE DETERMINATION OF THE MINIMAL LETHAL DOSE AND THE AVERAGE RATE OF UPTAKE OF G-STROPHANTHIN AND DIGITOXIN IN THE HEART-LUNG PREPARATION OF THE DOG

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Several attempts to determine the minimal lethal dose of certain cardiac glycosides for the cat heart-lung preparation have been made (1, 2, 3, 4); the average value of the minimal lethal dose (M.L.D.) of g-strophanthin, as determined by these investigators, varied between 1.5 and 2.6 micrograms per gram of cat heart. In all of these investigations the duration of the experiment did not exceed 50 minutes. Rothlin (3) has shown that when the experimental time was 30–40 minutes, the lethal dose (L.D.) of digitoxin was 10.5 micrograms, while an extension of this time to 80–90 minutes reduced the L.D. to 6.9 micrograms of digitoxin per gram of cat heart. In the intact animal a reduction of the rate of inflow of digitalis glycosides produced a prolongation of the experimental time and a reduction in the L.D. (5, 6). It is clear that the L.D. of cardioactive glycosides determined during short experimental periods or with high rates of administration cannot be the true M.L.D. So far as we know, no attempt has been made to establish the exact experimental conditions under which the true M.L.D. of cardiac glycosides may be determined on the heart-lung preparation (H.L.P.)

To determine the M.L.D. of a cardiac glycoside in the intact animal, two methods have been applied. The method used by Fromherz and Welsch (7) frequently needed observation periods exceeding 24 hours and was considered unsuitable for our purposes, since, even under the most favorable conditions, the life of the H.L.P. does not exceed 8–9 hours. Lendle (4), Mehnert (5) and Herre (8) used a method first introduced by Hauptstein (9). The characteristic feature of this method is the slow continuous infusion of the cardiac glycoside by means of a constant infusion pump. It was observed that when rates of administration were rapid, death would occur quickly and the L.D. would be high. When the rates of infusion were very slow, the L.D. would reach a value whereafter any further decrease of the rate of administration would merely lengthen the experimental time without any further lowering of the L.D. This value of the L.D. was considered to be the M.L.D. and the values obtained by this constant infusion method agree fairly well with those determined by the "timeless" method of Fromherz and Welsch (5). We have used the constant infusion method, since the M.L.D. could be determined within the period of viability of the dog H.L.P.; also, with this method, any destruction of cardioactive glycosides by the H.L.P. would be revealed by a gradual rise in the M.L.D. when the rate of administration of the drug was very slow (Hauptstein (9)).

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keep the ventricles beating until idioventricular rhythm set in, which in turn was followed by the characteristic fibrillation. Since mechanical stimulation was not very satisfactory, we employed 20 microgram doses of adrenaline to overcome this period of stoppage; in most instances only two and rarely three such doses were necessary.

In a series of experiments to be published later it will be shown that such amounts of adrenaline have no effect on either the M.L.D. or the average rate of uptake of g-strophanthin in the H.L.P. of the dog. Furthermore, from the data in table 1, it can be seen that adrenaline did not effect the M.L.D. of g-strophanthin, since in the experiments where adrenaline had to be used (exp. No. 18, 21, 23, 25, 26) the M.L.D. was 1.214 micrograms while in those experiments where adrenaline was not used (exp. No. 19, 20, 22, 24) the M.L.D. was 1.173 micrograms g-strophanthin per gram of heart.

When the experimental period exceeded 150 minutes, many of the hearts showed a marked infiltration of blood into the ventricular muscle, which was most apparent in the left ventricle and the apex of the heart. As a result of this infiltration, the heart weight was disproportionately high, and was corrected by approximating the true weight from the normal ratio of heart weight to body weight. In 38 dogs this ratio averaged 0.84 with a minimum and maximum of 0.58 and 1.12 respectively. Thus multiplying the body weight in kilograms by the factor 8.4 gave the approximate normal heart weight in grams.

Wood and Moe (14) have shown that in the H.L.P. edema of the heart seriously effects the determination of the heart weight. Furthermore they have shown that no accurate predicition of heart weight can be made from any formulae relating heart weight to body weight. According to Wood and Moe the only reliable method for determining heart weight in these preparations is by chloride space determinations, a method which unfortunately could not be used in the present study.

In four control experiments the heart was allowed to beat for 4–6 hours under the conditions described above, but no drug was infused. In none of the four experiments was there any gross infiltration or thickening of the ventricular muscle, although small hemorrhagic spots were observed in the region of the papillary muscles. These lesions were much less severe than those observed in hearts poisoned with cardioactive glycosides, and where the experimental time was of similar duration. In hearts where cardioactive glycosides were infused, and the experimental time was short, no such infiltration could be observed and we conclude that this infiltrative process is caused by the cardioactive glycosides acting over a long period of time. The question whether this infiltrative process has any effect on the M.L.D. cannot be answered definitely, but in the case of g-strophanthin, hearts showing an infiltration had a M.L.D. of 1.184 micrograms while in those experiments where no gross infiltration was apparent the M.L.D. was 1.201 micrograms per gram of heart, a difference which is less than the experimental error.

In some of the longer experiments there was a loss of circulating blood due to the development of edema of the lungs, but by using undiluted defibrinated blood only, and by keeping the inflation of the lungs at the smallest volume still compatible with a proper oxygenation of the blood, this edema was minimized. All experiments where the fluid loss exceeded 20 per cent of the original blood volume were discarded, excepting experiment 24 in table 2 where a fluid loss of about 35 per cent had to be recorded. This leakage would necessarily affect the determination of the M.L.D., but the error is probably not very great, since in most experiments the fluid loss was less then 15 per cent, and usually occurred only during the last 20 or 30 minutes of the experiment.

RESULTS. The influence of the rate of administration of g-strophanthin and digitoxin on the L.D. has been studied on the dog H.L.P. A total of 32 experiments were performed with g-strophanthin, of which 26 were considered successful, i.e., less than 20 per cent fluid loss. Twenty four out of 31 experiments were considered satisfactory with digitoxin. The results and data of these experiments are shown in tables 1 and 2.

The relationship between rate of administration and L.D. of g-strophanthin

Weese (1) has shown that in the H.L.P. of the cat, the heart will extract from the blood any quantity of g-strophanthin less than one M.L.D., but no more than one M.L.D. will be extracted regardless of the excess amount of glycoside in the blood. On the other hand, Genuit (10) and Genuit and Eschbach (11) have shown that rat and guinea pig hearts perfused with Ringer solution do not extract strophanthin quantitatively from the perfusion fluid. As an essential preliminary control we performed a number of experiments with the dog H.L.P.[2] and were able to show that the dog heart reacts in a manner similar to that of the cat in the quantitative uptake of glycoside. Since, regardless of the concentration, the dog H.L.P. binds any amount of cardiac glycoside not exceeding the M.L.D. it is possible to determine the *average rate of uptake* per gram heart per minute by dividing the M.L.D. by the experimental time (Herre (8)). By this procedure the specific affinity of cardiac glycosides for the heart can be accurately studied, since all other organs capable of absorbing the drug have been excluded (1).

In this paper we describe the influence of the rate of administration of g-strophanthin and digitoxin on the lethal dose, experimental time, destruction and rate of uptake in the H.L.P.

MATERIALS AND METHODS. The cardiac glycosides employed were crystalline g-strophanthin Thoms (Merck) and crystalline digitoxin (Hoffmann La Roche[3]). According to the manufacturers, g-strophanthin Thoms contains 20 per cent water of crystallization (12). All values given in this paper have been calculated on the basis of anhydrous g-strophanthin. Stock solutions containing 0.5 per cent g-strophanthin or digitoxin in 95 per cent ethyl alcohol were diluted in 0.9 per cent saline. The dilutions in saline were varied from 1:5000 to 1:1,000,000 for g-strophanthin, and from 1:25,000 to 1:200,000, for digitoxin.

All constant infusions were made with a small Marriotte tube into the venous end of the heart-lung circuit and the rate of administration was regulated at the beginning of each experiment by means of a small screw clamp. All though the experiment great care was taken to keep the rate of inflow constant and, to this end, the number of drops of fluid infused per unit of time was frequently checked. The simplicity of this method of infusion does not detract from the accuracy of the results obtained, as a later series of experiments with a more elaborate method of constant infusion did not show any significant differences from the results described below.

The H.L.P. was prepared by the technic of Knowlton and Starling (13), using dogs weighing between 2.15 and 11.6 kg. Anesthesia was induced with ether and followed by 90 mg. per kg. of chloralose given intravenously. In each of the experiments described, all controllable factors were as follows: blood temperature, 38.9–39.2°C.; blood volume, 850–900 cc.; resistance, 75 mm. of mercury; output of the left heart, 400–450 cc. per minute; and the work of the left heart, 0.65–0.72 kg.-m. per minute. The heart rate was not controllable, but varied between 138–192 beats per minute. Right venous pressure varied between 2 and 5 cm. of water. All preparations showing irregular rhythms, early insufficiency, or severe edema of the lungs were discarded. The end point of the experiment was the appearance of ventricular fibrillation, and the infusion of the drug was continued until such fibrillation began.

In experiments of long duration, we occasionally observed that during the period of complete block the ventricles would beat at a rate of 5–10 beats per minute or would stop completely. This stoppage was clearly *not* the end point since mechanical stimulation would tion of a L.D. of 3.44 micrograms per gram heart. The blood from this preparation, when added to a second heart, contained enough glycoside to kill the second preparation within a period of 64 minutes. On the other hand, the blood from a preparation where no overtitration had taken place did not poison the heart when the blood was transferred to the second H.L.P. (Experiment 1, table 3).

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[2] Dogs were employed for our experiments because they were more easily obtained than cats.

[3] Kindly supplied by Hoffmann La Roche, Basel, Switzerland.

TABLE 2

The effect of the rate of administration of digitoxin on the lethal dose, rate of uptake and experimental time in the dog heart-lung preparation

EXP. NO.	WEIGHT OF DOG	BLOOD TEMPERATURE	HEART WEIGHT IN GRAMS		RATE OF ADMINISTRATION, MICROGRAM PER GRAM HEART, PER MINUTE	LETHAL DOSE, MICROGRAM PER GRAM HEART	EXPERIMENTAL TIME	RATE OF UPTAKE, MICROGRAM PER GRAM HEART, PER MINUTE	RATE OF UPTAKE PER CENT OF MINIMAL LETHAL DOSE	REMARKS
			Uncorrected	Corrected						
	kg.	°C.					minutes			
1	6.1	39	55		3.703	100.1	27	0.219	3.71	
2	4.9	39	41		0.721	44.7	62	0.0953	1.61	
3	6.0	39	54		0.551	37.4	68	0.087	1.47	
4	2.45	39	27		0.54	41.06	76	0.0777	1.30	Young animal
5	7.12	39.1	51.2		0.504	33.77	67	0.0882	1.49	
6	5.3	39	49.7		0.472	32.56	69	0.0856	1.45	
7	3.42	38.8	39		0.458	35.64	78	0.0758	1.28	Young animal
8	2.15	39	30		0.443	38.08	86	0.068	1.16	Young animal
9	4.42	39	32.5		0.413	33.16	81	0.0729	1.23	
10	5.7	39	47.2		0.318	26.88	83	0.0712	1.20	Slight fluid loss
11	5.1	39.1	46		0.294	23.85	81	0.0729	1.23	
12	4.52	39.1	48.5		0.276	22.68	82	0.0723	1.22	
13	7.12	39	51.2		0.247	19.12	78	0.0758	1.28	Fluid loss 50 cc.
14	5.50	39.2	42.5		0.166	17.9	108	0.0549	0.92	
15	6.49	39	57.7		0.132	14.38	109	0.0542	0.92	
16	7.60	39	69.0		0.0798	10.37	131	0.0451	0.76	Fluid loss 100 cc.
17	7.12	39	66		0.0761	9.06	119	0.0488	0.84	
18	7.30	39	65.5		0.0521	8.86	170	0.0348	0.59	Fluid loss 50 cc.
19	7.93	39	87	67	0.040	6.53	163	0.0356	0.61	Adrenaline 1 × 20 micrograms
20	5.03	39.1	71		0.0338	6.19	183	0.0332	0.56	Fluid loss 100 cc.
21	5.6	39	75.5	47	0.0265	6.28	237	0.0249	0.42	Fluid loss 150 cc.
22	5.9	39	35		0.0236	5.43	230	0.0257	0.43	Fluid loss 150 cc. Adrenaline 2 × 20 micrograms
23	7.4	39	74	62	0.019	5.44	286	0.0206	0.35	Fluid loss 200 cc. Adrenaline 2 × 20 micrograms
24	5.95	39.1	84	50	0.0152	5.75	375	0.016	0.26	Fluid loss 250–300 cc. Adrenaline 3 × 10 micrograms

When the rate of administration is slow, both g-strophanthin and digitoxin showed a flattening of their respective curves; further reduction of the rate of administration did not reduce the L.D. On the basis of these experiments we would designate as *optimal rate of administration* (O.R.A.), that rate below which and digitoxin has been graphically represented in figure 1. With the higher rates of administration employed, the L.D. is about ten times the M.L.D. Since, in the heart-lung system, the heart is the only organ which can bind cardioactive

TABLE 1

The effect of the rate of administration of g-strophanthin on the lethal dose, rate of uptake and experimental time in the dog heart-lung preparation

EXP. NO.	WEIGHT OF DOG	TEMPERATURE	HEART WEIGHT IN GRAMS		RATE OF ADMINISTRATION, MICROGRAM PER GRAM HEART, PER MINUTE	LETHAL DOSE, MICROGRAM PER GRAM HEART	EXPERIMENTAL TIME	RATE OF UPTAKE, MICROGRAM PER GRAM HEART, PER MIN.	RATE OF UPTAKE, PER CENT OF MINIMAL LETHAL DOSE	REMARKS
			Uncorrected	Corrected						
	kilograms	°C.					*minutes*			
1	7.45	39	60.5		0.830	14.93	18	0.066	5.55	
2	8.20	39.1	75.5		0.713	9.97	14	0.085	7.14	
3	11.60	39	68		0.619	10.23	16	0.074	6.25	
4	5.6	39	43.5		0.592	10.5	18	0.066	5.55	
5	5.85	39.2	62.5		0.445	6.12	14	0.085	7.14	
6	5.50	39	47.5		0.433	9.1	21	0.056	4.76	
7	9.41	39	66.5		0.337	8.1	24	0.048	4.17	
8	5.51	39.1	41		0.219	6.14	28	0.042	3.57	
9	4.70	39.2	41		0.141	5.62	40	0.030	2.5	
10	5.95	39	56		0.118	4.94	42	0.028	2.37	
11	6.25	39	43.5		0.0972	4.38	45	0.027	2.22	Fluid loss about 50 cc.
12	6.60	38.8	59		0.065	3.57	55	0.022	1.81	
13	7.20	39.2	67.5		0.048	2.89	60	0.0195	1.66	
14	6.15	38.8	53		0.0311	2.23	72	0.017	1.39	Fluid loss about 50 cc.
15	5.00	39	58.5		0.028	1.87	67	0.018	1.49	
16	5.22	39	61	44	0.028	1.99	72	0.017	1.39	
17	5.20	39.2	55		0.0194	1.9	98	0.012	1.02	
18	3.95	39.1	34.5		0.010	1.39	139	0.0085	0.705	Fluid loss about 50 cc. adrenaline 1. × 30 micrograms
19	6.30	39.2	70	53	0.0098	1.23	126	0.0097	0.79	Fluid loss about 50 cc.
20	5.30	39	50		0.0095	1.011	107	0.0111	0.93	
21	5.45	39	46.5		0.0082	1.068	129	0.0091	0.77	Adrenaline 1 × 20 micrograms
22	3.22	39.1	28.5		0.0076	1.334	174	0.0068	0.57	Fluid loss 80–100 cc.
23	7.4	39.2	107	62	0.0069	1.501	213	0.0055	0.47	Adrenaline 2 × 20 micrograms. Fluid loss 100 cc.
24	10.9	39.1	148	92	0.00667	0.901	235	0.0051	0.42	Fluid loss 100 cc.
25	8.3	39	95	70	0.00612	1.134	195	0.0062	0.51	Adrenaline 2 × 20 micrograms. Fluid loss 150 cc.
26	7.01	39.1	91	59	0.0041	1.154	280	0.0042	0.36	Adrenaline 2 × 20 micrograms. Fluid loss ab. 200 cc.

glycosides (1), these high lethal doses are due to the inability of the heart to bind all the drug infused. A proof that the excess glycoside is still in the blood is found in experiment 2 of table 3. Here an overtitration resulted in a determinaheart. These minimal doses are based on the correction of the heart weight described under methods.

Rothlin (3) has shown that a lengthening of the experimental time lowers the L.D. of digitoxin for the H.L.P. of the cat. Our results confirm and extend this finding for g-strophanthin and digitoxin for the dog H.L.P. (fig. 2). However, the lowering of the L.D. continued until an experimental time was reached, beyond which any further extension did not change the L.D. Under our experimental conditions, the minimal experimental time needed for determining the M.L.D. was about 120 minutes for g-strophanthin, and 230 minutes for digitoxin. Weese and Rothlin did not use an experimental time greater than 90 minutes, and thus presumably did not determine the true M.L.D.

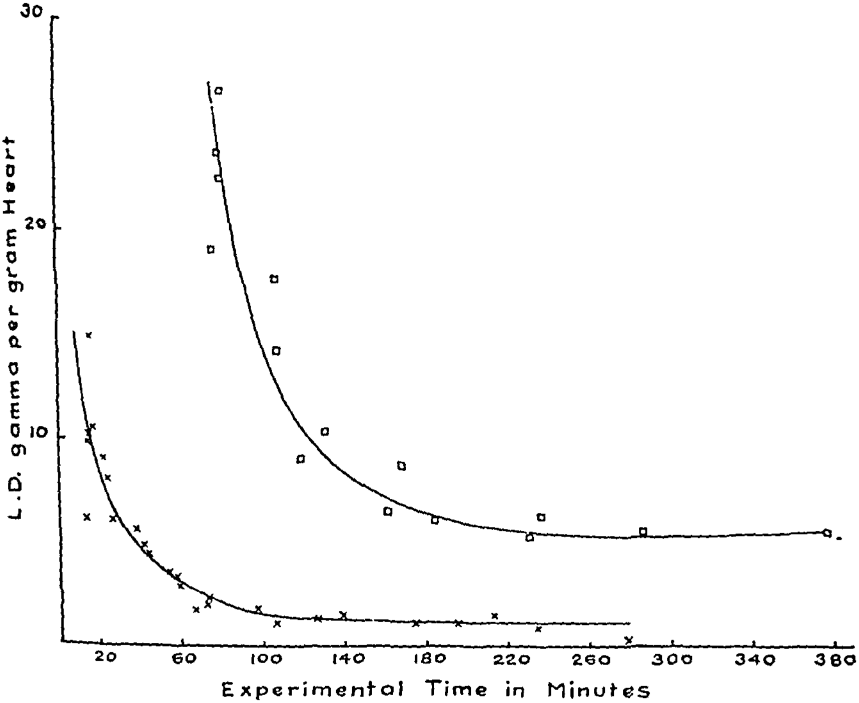

FIG. 2. DOG HEART-LUNG PREPARATION

The relation of experimental time to the lethal dose of g-strophanthin and digitoxin. (Gamma = microgram)

□————□ Digitoxin ×————× g-strophanthin

Weese (1), while studying the action of scillaren on the H.L.P. of the cat, noticed that this substance was inactivated fairly rapidly. Any destruction of either g-strophanthin or digitoxin in the dog H.L.P. would manifest itself by a gradual rise in the M.L.D. when rates of inflow are less than the optimal. As can further reduction fails to lower the L.D. This O.R.A. has a characteristic value for each of the two glycosides studied, and under our experimental conditions is about 0.012 microgram and 0.028 microgram per gram heart per minute for g-strophanthin and digitoxin respectively. The optimal rate of administration should be differentiated from the term *critical rate of administration* (Kritische Einlaufgeschwindigkeit) of Heubner and Nyary (15), since the latter denotes the rate of administration at which elimination and rate of administration are equal.

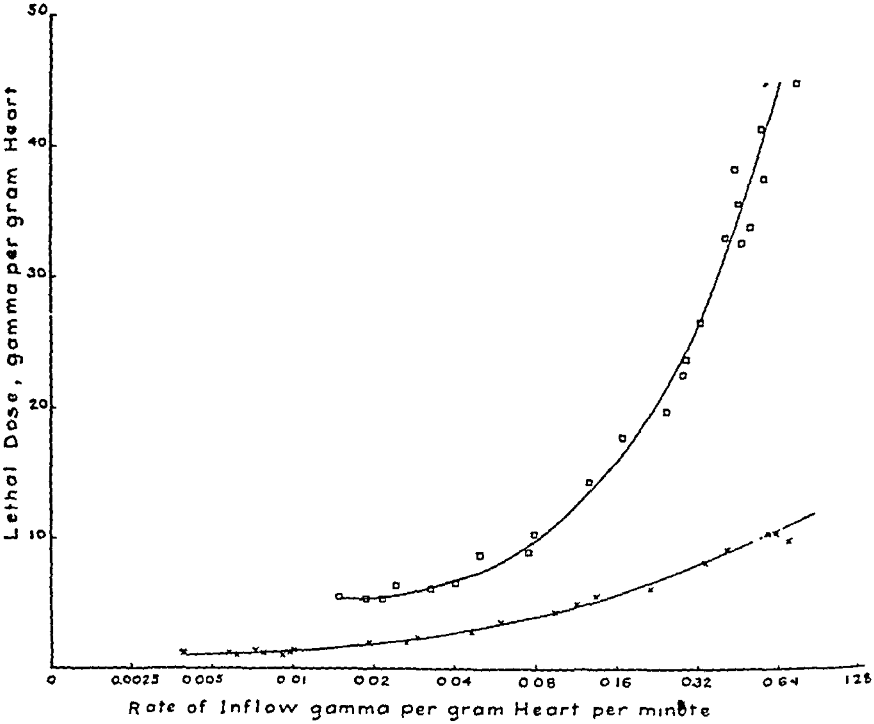

FIG. 1. DOG HEART-LUNG PREPARATION

The relation of rate of inflow of g-strophanthin and digitoxin to the lethal dose. (Gamma = microgram)

□————□ Digitoxin ×————× g-strophanthin

A large number of the experiments in which we tried to determine the M.L.D. of g-strophanthin and digitoxin had to be discarded, because prolonged experimental time produced edema of the lungs which could neither be prevented nor controlled. In 12 experiments with g-strophanthin, 8 were successful, while with digitoxin only 4 out of 9 experiments could be considered satisfactory. From these successful experiments we determined an average M.L.D. of 1.184 micrograms of g-strophanthin and 5.95 micrograms of digitoxin per gram of dog can be detected in the blood if this is transferred to a second preparation. Experiment 3 is similar to experiment 2, but the results are quantitative. To 720 cc. of blood in the first preparation, 95 micrograms of g-strophanthin were added. This heart weighed 53 grams; therefore, had the M.L.D. been 1.18 micrograms, it should have bound 62 micrograms of g-strophanthin, thus leaving approximately 33 micrograms unbound in the 720 cc. of blood. To a second preparation we added 520 cc. of the blood from the first preparation but in order to stop the second heart it was necessary to add an additional 54 micrograms g-strophanthin. The second heart weighed 69 grams, therefore its M.L.D. could be expected to be approximately 81.5 micrograms of g-strophanthin. Since 54 micrograms of the drug were added to the blood of the second heart, it may be concluded that 26.5 micrograms of g-strophanthin were present in the 520 cc. of blood from the first heart. Hence, the 720 cc. of blood from H.L.P. No. 1 must have contained 36.5 micrograms, a value which agrees well with the calculated amount of 33 micrograms. It may be concluded, therefore, that the dog H.L.P. is similar to that of the cat, in that it binds no more than one M.L.D. of g-strophanthin, regardless of excess concentration.

Since, regardless of the concentration, the heart only binds an amount of cardioactive glycosides equal to the M.L.D., we have calculated the average rate of uptake by dividing the M.L.D. by the experimental time. This rate of uptake is the amount of glycoside bound by one gram of heart in one minute. It should be remembered, however, that the rate of uptake may not be the same during all the stages of the poisoning process. Gehlen (16), from purely mathematical considerations, believes that the uptake of digitalis glycosides by the heart is more rapid during the earlier than during the later stages of this poisoning process. The binding of cardiac glycosides by the heart is a complex mechanism which has been divided by some investigators into an adsorptive and a chemical phase (17). Our term *average rate of uptake* is the resultant of the processes of transfer of the glycoside from the blood to the cardiac cells and the various reactions which they undergo in the cell to produce the characteristic pharmacological manifestations.

In fig. 3 we have plotted the average rate of uptake against the rate of administration. It can be seen that with a reduction in the rate of administration, the rate of uptake decreases. At the *optimal rate of administration* the average rate of uptake equals the rate of administration. The average rate of uptake of digitoxin, expressed in micrograms, is higher than that of g-strophanthin. If, however, the rate of uptake is expressed in per cent of the M.L.D., g-strophanthin has a higher rate of uptake. This is to be expected, since the speed of reaction of g-strophanthin is greater (1), and the M.L.D. less, than that of digitoxin.

DISCUSSION. The results of this investigation on the H.L.P. of the dog clearly show that, within certain limits, the L.D. of a cardioactive glycoside is dependent on its rate of administration. If the M.L.D. is being determined by continuous infusion, it is necessary to extend the experimental periods to 120 minutes for g-strophanthin and 240 minutes for digitoxin. The high values of be seen from our results there is no rise in the M.L.D. under such conditions, and it is likely that neither g-strophanthin nor digitoxin have been destroyed within the experimental periods used by us.

A number of experiments designed to see whether the dog H.L.P. binds only an amount of g-strophanthin equal to the M.L.D., regardless of the concentration, have been tabulated in table 3. The technic employed by us is similar to that used by Weese for studying the same point in the H.L.P. of the cat (1). From the first experiment it can be concluded that the glycoside infused was sufficient only to poison the first preparation, since the blood transferred to the second heart showed no digitalis action. From experiment 2 it can be concluded that when a rate of administration greater than the optimal is used, a g-strophanthin

TABLE 3

Passage experiments with g-strophanthin in the dog heart-lung preparation

EXPERIMENT I

Heart No. 1 = 34.5 grams. Blood volume = 900 cc.

9:06 Continuous infusion of 1:500,000 g-strophanthin at a rate of 0.0101 microgram per gram heart per minute.
11:15 Cardiac arrest in 139 minutes. L.D. = 1.390 micrograms g-strophanthin per gram of heart.

Heart No. 2 = 49 grams. (corrected)

12:06 850 cc. of blood from Heart No. 1 was added.
No change in cardiac action.
1:44 Started infusion of g-strophanthin 1:500,000 at a rate of 0.0091 microgram per gram heart per minute.
3:08 Irregularities set in.
3:51 Ventricular fibrillation. L.D. = 1.16 micrograms of g-strophanthin per gram heart.

EXPERIMENT II

Heart No. 1 = 43.0 gram. Blood volume = 850–900 cc.

9:21 Infusion g-strophanthin, 1:100,000, at a rate of 0.06 microgram per gram heart per minute.
9:52 Irregularities set in.
10:19 Ventricular fibrillation. L.D. = 3.49 micrograms per gram heart.

Heart No. 2 = 53 grams. Blood volume = 200 cc.

11:20 Added 870 cc. of blood from heart No. 1.
11:55 Irregularities.
12:24 Ventricular fibrillation.

EXPERIMENT III

Heart No. 1 = 53 grams. Blood volume = 720 cc.

1:51 Added 96 micrograms g-strophanthin.
2:54 Ventricular fibrillation.

Heart No. 2 = 69 grams. Blood volume = 300 cc.

3:41 Added 520 cc. of blood from Heart No. 1.
4:41 Started infusion of 1:500,000 g-strophanthin at a rate of 0.00477 microgram per gram heart per minute.
6:50 20 micrograms adrenaline injected.
7:25 Ventricular fibrillation. L.D. 0.783 microgram per gram heart.

phanthin, and between 0.37 and 0.5 mg. per kg. of digitoxin (20, 21). The lowest of these values are probably the closest to the true M.L.D., and have been used in our calculations. Since in the dog, the heart is on the average equal to 0.84 per cent of the body weight, the amount of glycoside bound by 8.4 grams of heart should be compared with the M.L.D. per kg. of the intact dog. For g-strophanthin we found that 11.8 per cent, and for digitoxin 13.5 per cent of the M.L.D. of the intact animal is bound by the heart. These values are somewhat higher than those calculated by Weese (1) and Rothlin (3) for the cat, but their values are based on the Hatcher-Magnus dose, and thus the values of the percentage utilization by the cat heart are too low. Since in the dog, the ratio of heart weight to body weight is about twice that in the cat, some species differences in this partition between the heart and other organs would be most interesting, but a final decision on this point must await the accurate determination of the M.L.D. of these glycosides in the cat H.L.P. and the intact dog.

While studying the influence of caffeine on the L.D. of strophanthin in the cat H.L.P. Kohn (22) expressed the opinion that the reduction of the L.D. by caffeine was due to an increase in the permeability of the cardiac cell to cardiac glycosides. Weese and Wiegand (23) have shown that a large dose of caffeine (5 mg. per gram heart) markedly lowered the L.D. of g-strophanthin in the cat H.L.P. Both Weese and Kohn were probably working with short experimental times, so that values higher than the M.L.D. were being determined. It is conceivable that at rates higher than the *optimal rate of administration*, a modification of the L.D. may be due either to a change in the rate of uptake or a change in the true M.L.D. Thus caffeine may have either increased the rate of uptake or decreased the M.L.D. To decide which of these two factors is operating, one would have to determine the M.L.D. and also the L.D. and experimental time at various rates of administration, with and without caffeine. From this data it would be possible to calculate the rate of uptake and thus determine whether the change in the L.D. is due to a reduction in the M.L.D. or an increase in the uptake. A similar reasoning could be applied to any substance which has the ability to change the L.D. of a cardiac glycoside in the H.L.P., when rates greater than the optimal rate of administration are being used.

SUMMARY

The effect of rate of administration of g-strophanthin and digitoxin on the L.D., experimental time, destruction and rate of uptake has been studied in the H.L.P. of the dog.

With high rates of administration the L.D. is high. This is due to an over-titration resulting from the inability of the heart to bind all the glycoside offered per unit of time. To determine the M.L.D., rates equal to or lower than the *optimal rates of administration* have to be employed. The M.L.D. as determined under our experimental conditions is 1.18 micrograms g-strophanthin (0.002 micromols) and 5.95 micrograms of digitoxin (0.008 micromols).

In our experiments it was not possible to detect any destruction of either g-strophanthin or digitoxin by the H.L.P. of the dog.

the M.L.D. of some cardioactive glycosides determined previously (1, 2, 3, 4) are probably due to the fact that these investigators were not extending their experimental periods long enough. Moe (18) has determined the minimal irregularity dose (M.I.D.) for some cardiac glycosides in the dog H.L.P. His value for the M.I.D. for g-strophanthin was 0.37 micrograms per gram heart. According to Krueger and Unna (19) the M.I.D. is about 60 per cent of the M.L.D. in the intact anesthetized cat. If the same relationships should hold for the dog H.L.P., the M.L.D. of g-strophanthin would be 0.617 micrograms per gram of heart. This value is about 50 per cent lower than the one determined by us. It is not possible to discuss any further this discrepency since the methods of Moe are somewhat different from those described in this paper.

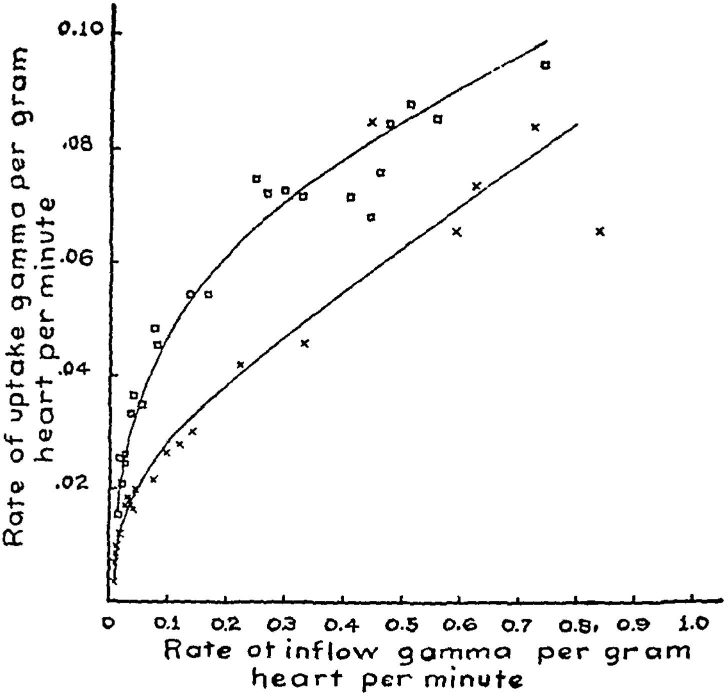

FIG. 3. DOG HEART-LUNG PREPARATION

The relation of the rate of inflow of g-strophanthin and digitoxin to the rate of uptake. (Gamma = microgram)

□————□ Digitoxin ×————× g-strophanthin

From our data the partition between the heart and other organs in the intact dog can now be calculated. For comparative purposes the most reasonable values to be used are the M.L.D. of both the intact animal and the H.L.P. In the intact dog the reported lethal doses vary between 0.084 and 0.19 for g-strophanthin, and between 0.37 and 0.5 mg. per kg. of digitoxin (20, 21). The lowest of these values are probably the closest to the true M.L.D., and have been used in our calculations. Since in the dog, the heart is on the average equal to 0.84 per cent of the body weight, the amount of glycoside bound by 8.4 grams of heart should be compared with the M.L.D. per kg. of the intact dog. For g-strophanthin we found that 11.8 per cent, and for digitoxin 13.5 per cent of the M.L.D. of the intact animal is bound by the heart. These values are somewhat higher than those calculated by Weese (1) and Rothlin (3) for the cat, but their values are based on the Hatcher-Magnus dose, and thus the values of the percentage utilization by the cat heart are too low. Since in the dog, the ratio of heart weight to body weight is about twice that in the cat, some species differences in this partition between the heart and other organs would be most interesting, but a final decision on this point must await the accurate determination of the M.L.D. of these glycosides in the cat H.L.P. and the intact dog.

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In our experiments it was not possible to detect any destruction of either g-strophanthin or digitoxin by the H.L.P. of the dog.

The dog heart in the H.L.P. binds only an amount of cardiac glycoside equal to the M.L.D. regardless of the concentration in the blood. It was thus possible to calculate the *average rate of uptake* of g-strophanthin and digitoxin by dividing the M.L.D. by the experimental time. The *average rate of uptake* thus determined increased with an increase in the rate of administration.

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FURTHER STUDY OF SOME 1-SUBSTITUTED THEOBROMINE COMPOUNDS

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In a previous communication (1), a comparison was made of the action of caffeine and 1-ethyl theobromine. Although in certain respects the latter appeared more potent than caffeine, particularly in animals, the degree of difference between the 2 compounds was not great enough apparently to be of practical interest. However, the results were sufficiently encouraging to warrant a further study of other analogues. Included in this investigation were the ethyl, *n*-propyl, *n*-butyl, *iso*amyl, allyl, methallyl, crotyl, and methoxyethyl theobromines. The well-known products, caffeine and, in certain instances, theophylline, theobromine, *d*-amphetamine sulfate, lobeline sulfate, and nikethamide, were also employed for the sake of comparison. We are greatly indebted to Dr. L. P. Kyrides, of the Monsanto Chemical Company, who supplied us with all the derivatives of theobromine.

The majority of the chemicals tested are not new. Van der Slooten (2) was the first to prepare successfully 1-alkyl theobromine derivatives. In spite of many reports describing preparation of these substances, pharmacologic studies have been very limited. Bergell and Richter (3) tested the diuretic action of several 1-alkyl theobromines, and Ritz (4) investigated the pharmacology of 1-allyl theobromine. No references have been made in literature to 3 compounds of the present series, namely, 1-methallyl, 1-crotyl, and 1-methoxyethyl theobromine.

Convulsive action and acute toxicity in mice. The median convulsive doses and median lethal doses ± standard errors were determined by injection into the tail veins of starved albino mice. Computations were made according to the method of Bliss (5), the results being given in table 1. The compounds showed the following order of decreasing toxicity: allyl > ethyl > crotyl > methyl (caffeine) propyl > butyl > *iso*amyl > methallyl > methoxyethyl. The convulsive potency had the same order except that the butyl and *iso*amyl derivatives appeared to have no convulsive action whatsoever. The type of convulsions was similar in all instances, namely, tetanic. Death either occurred within a few minutes or not at all.

The results show that with increasing length of the saturated aliphatic side chain, the activity increases up to the ethyl derivative and thereafter a rapid decrease follows. The most potent compound of all has the unsaturated allyl chain. Addition of a methyl group to the allyl chain markedly lowers toxicity and convulsive action. The same is true when a methoxy radical is attached to the ethyl group.

Feeding experiments in rats. Since we were particularly interested in stimulation of the central nervous system, only 2 of the more convulsive compounds were tested for chronic toxicity. Caffeine was included for comparison. Young albino rats weighing about 80 grams were given a diet in which propyl theobromine, allyl theobromine, or caffeine was incorporated in various percentages (6). Six groups of 5 rats each were tested on each compound, each group receiving a different percentage, but the same percentages were used for each chemical—namely, 0.02, 0.035, 0.07, 0.1, 0.3, and 0.5 per cent. The test period lasted 28 days.

There was an inhibition of weight gains—much greater with allyl theobromine than with the other 2 substances. The effect of propyl theobromine and caffeine appeared to be equal. Little or no effect on weight was noted with these substances in the amount of 0.1 per cent or less in the diet, as compared with the average weight gain of a separate group of 4 control rats without medication. In the 0.5 per cent group which received allyl theobromine, 1 rat died in 26 days.

TABLE 1

The median convulsive and median lethal doses of 1-substituted theobromine compounds by intravenous injection into mice

THEOBROMINE DERIVATIVE	NUMBER OF ANIMALS USED	CD_{50} ± S. E.	LD_{50} ± S. E.
		mg per kg.	*mg. per kg.*
Caffeine	35	84.4 ± 4.9*	100.9 ± 6.7*
Ethyl	40	54.1 ± 2.0*	61.0 ± 2.4*
Propyl	40	96.3 ± 4.2	125.8 ± 5.1
Butyl	30	non-convulsive	167.0 ± 15.2
*Iso*amyl	30	non-convulsive	about 200.0
Allyl	50	35.7 ± 0.8	40.0 ± 1.0
Methallyl	65	119.0 ± 5.6	254.0 ± 14.6
Methoxyethyl	40	240.0 ± 6.8	272.0 ± 7.3
Crotyl	70	56.1 ± 1.8	94.5 ± 4.7

* Previously reported (1).

At the end of the test period, all rats fed the 0.5 per cent diet were submitted to necropsy. The findings were essentially nonspecific, except that the rat which died on the allyl theobromine diet showed fatty metamorphosis of the liver. In addition, mild hypertrophy of the thyroid gland was noted in 60 per cent of the rats which were fed propyl theobromine regardless of the dose. Unfortunately, the thyroids were not examined in the other 2 series.

Stimulation of motor activity of rats. Using the method of Schulte, Tainter, and Dille (7) of measuring the amount of activity of adult rats placed in spring-suspended cages, the stimulating actions of these compounds were compared. Administration of all substances was by subcutaneous injection. Theophylline and *d*-amphetamine sulfate were included in the study for purposes of comparison. The results are to be seen in table 2. Caffeine, or, chemically, methyl theobromine, produced the greatest stimulation of the theobromine series. The ethyl compound was next most potent followed by the allyl, propyl, and crotyl derivatives. The remaining substances caused little or no change in activity of the animals. In control experiments, untreated animals registered no activity, or, at the most, 1 to 2 revolutions in 4 hours.

Schulte and his co-workers (7) believe that their method measures the degree of stimulation of the higher centers of the brain. Convulsive action of theobromine derivatives may be indicative more of spinal cord stimulation than of stimulation of higher centers, particularly since convulsions produced by these compounds were tetanic in nature (8). Since caffeine in our work was more potent than the allyl derivatives by the cage-activity test, and the reverse held true for the convulsive effect, the potency of each compound may be variable in different parts of the central nervous system. Schulte and associates found no increase of activity by their method when rats were given metrazol or picrotoxin, although these drugs are well-recognized as strong central nervous system stimulants.

TABLE 2

Comparative effects of theobromine derivatives on activity of rats in spring-suspended cages

THEOBROMINE DERIVATIVE	NUMBER OF RATS	TOTAL NUMBER OF REVOLUTIONS DURING 4 HOURS. AVERAGE PER RAT				
		Dose (mg. per kg)				
		5	10	20	40	80
Caffeine	25	8.0	21.0	25.8	5.0	16.6
Ethyl	25	8.0	14.2	15.0	10.8	14.8
Propyl	25	3.2	12.2	9.0	13.0	12.8
Butyl	25	1.4	5.4	3.4	2.6	3.2
*Iso*amyl	25	3.0	5.4	3.4	2.6	4.0
Allyl	25	5.0	14.2	10.2	9.0	22.6
Methallyl	25	3.0	5.6	2.6	1.2	2.6
Crotyl	25	3.2	5.0	6.2	6.6	13.6
Methoxyethyl	25	4.6	5.0	5.6	5.2	9.4
Theophylline	75	9.1	11.7	41.0	25.1	25.3
		1	2	3	4	5
d-Amphetamine Sulfate	50	15.5	51.0	61.0	25.5	49.8

Surprisingly, theophylline produced greater motor activity than caffeine. This is in contrast to the established superiority of caffeine over theophylline as a central nervous system stimulant in man. *d*-Amphetamine sulfate was distinctly more stimulating than any of the compounds tested.

Respiratory stimulation in dogs. Under deep anesthesia with phenobarbital sodium, the volume of expired air of dogs was recorded continuously. Details of the method were given in a previous communication (1). An attempt was made to depress respiration of each animal to approximately the same degree. The volumes of expired air for 30 minutes before and after intravenous injection of

Feeding experiments in rats. Since we were particularly interested in stimulation of the central nervous system, only 2 of the more convulsive compounds were tested for chronic toxicity. Caffeine was included for comparison. Young albino rats weighing about 80 grams were given a diet in which propyl theobromine, allyl theobromine, or caffeine was incorporated in various percentages (6). Six groups of 5 rats each were tested on each compound, each group receiving a different percentage, but the same percentages were used for each chemical—namely, 0.02, 0.035, 0.07, 0.1, 0.3, and 0.5 per cent. The test period lasted 28 days.

There was an inhibition of weight gains—much greater with allyl theobromine than with the other 2 substances. The effect of propyl theobromine and caffeine appeared to be equal. Little or no effect on weight was noted with these substances in the amount of 0.1 per cent or less in the diet, as compared with the average weight gain of a separate group of 4 control rats without medication. In the 0.5 per cent group which received allyl theobromine, 1 rat died in 26 days.

TABLE 1

The median convulsive and median lethal doses of l-substituted theobromine compounds by intravenous injection into mice

THEOBROMINE DERIVATIVE	NUMBER OF ANIMALS USED	CD_{50} ± S. E.	LD_{50} ± S. E
		mg per kg.	*mg. per kg.*
Caffeine.	35	84.4 ± 4.9*	100.9 ± 6.7*
Ethyl	40	54.1 ± 2.0*	61.0 ± 2.4*
Propyl	40	96.3 ± 4.2	125.8 ± 5.1
Butyl	30	non-convulsive	167.0 ± 15.2
*Iso*amyl	30	non-convulsive	about 200.0
Allyl	50	35.7 ± 0.8	40.0 ± 1.0
Methallyl	65	119.0 ± 5.6	254.0 ± 14.6
Methoxyethyl	40	240.0 ± 6.8	272.0 ± 7.3
Crotyl	70	56.1 ± 1.8	94.5 ± 4.7

* Previously reported (1).

At the end of the test period, all rats fed the 0.5 per cent diet were submitted to necropsy. The findings were essentially nonspecific, except that the rat which died on the allyl theobromine diet showed fatty metamorphosis of the liver. In addition, mild hypertrophy of the thyroid gland was noted in 60 per cent of the rats which were fed propyl theobromine regardless of the dose. Unfortunately, the thyroids were not examined in the other 2 series.

Stimulation of motor activity of rats. Using the method of Schulte, Tainter, and Dille (7) of measuring the amount of activity of adult rats placed in spring-suspended cages, the stimulating actions of these compounds were compared. Administration of all substances was by subcutaneous injection. Theophylline and *d*-amphetamine sulfate were included in the study for purposes of comparison. The results are to be seen in table 2. Caffeine, or, chemically, methyl theobromine, produced the greatest stimulation of the theobromine series. The ethyl compound was next most potent followed by the allyl, propyl, and crotyl derivatives. The remaining substances caused little or no change in activity of the animals. In control experiments, untreated animals registered no activity, or, at the most, 1 to 2 revolutions in 4 hours.

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TABLE 2

Comparative effects of theobromine derivatives on activity of rats in spring-suspended cages

THEOBROMINE DERIVATIVE	NUMBER OF RATS	TOTAL NUMBER OF REVOLUTIONS DURING 4 HOURS. AVERAGE PER RAT				
		Dose (mg. per kg.)				
		5	10	20	40	80
Caffeine	25	8.0	21.0	25.8	5.0	16.6
Ethyl	25	8.0	14.2	15.0	10.8	14.8
Propyl	25	3.2	12.2	9.0	13.0	12.8
Butyl	25	1.4	5.4	3.4	2.6	3.2
*Iso*amyl	25	3.0	5.4	3.4	2.6	4.0
Allyl	25	5.0	14.2	10.2	9.0	22.6
Methallyl	25	3.0	5.6	2.6	1.2	2.6
Crotyl	25	3.2	5.0	6.2	6.6	13.6
Methoxyethyl	25	4.6	5.0	5.6	5.2	9.4
Theophylline	75	9.1	11.7	41.0	25.1	25.3
		1	2	3	4	5
d-Amphetamine Sulfate	50	15.5	51.0	61.0	25.5	49.8

Surprisingly, theophylline produced greater motor activity than caffeine. This is in contrast to the established superiority of caffeine over theophylline as a central nervous system stimulant in man. *d*-Amphetamine sulfate was distinctly more stimulating than any of the compounds tested.

Respiratory stimulation in dogs. Under deep anesthesia with phenobarbital sodium, the volume of expired air of dogs was recorded continuously. Details of the method were given in a previous communication (1). An attempt was made to depress respiration of each animal to approximately the same degree. The volumes of expired air for 30 minutes before and after intravenous injection of the test substance were compared. Using several dogs for each drug, the average per cent increase of expired air volume was calculated. Only 1 compound could be tested in each animal owing to tachyphylaxis. The well-known respiratory stimulants, lobeline sulfate and nikethamide, were included for comparison. Results are given in table 3. Propyl theobromine was much more stimulating than any substance tested. The order of potency was propyl > crotyl > methallyl > butyl > *iso*amyl >ethyl > allyl > methyl (caffeine) > methoxyethyl. Addition of a methyl group to the allyl radical increased the respiratory action but the methoxy group depressed the activity of the ethyl chain. Lobeline sulfate was between the butyl and *iso*amyl compounds in stimulating power, while nikethamide was slightly weaker than caffeine.

Diuretic action. Albino *rats* were studied by the technic of Lipschitz, Hadidian, and Kerpcsar (9). The animals were given 25 cc. per kg. of 0.9 per cent sodium chloride solution by stomach tube at the start of the experiment. Control animals received saline solution alone, while test rats were given the compounds dissolved in the sodium chloride solution. Each dose was tested on 4 rats, all doses of a given compound being run the same day. This procedure was repeated on another day, and the average calculated for the 8 animals on each dose. The diuretic effect was ascertained by the ratio of urine volumes between test animals and control rats. Caffeine was found to have a diuretic ratio of 2.45 in the dose of 0.2 millimole per kg., being greater than that of any of the theobromine derivatives. Crotyl theobromine had little or no diuretic action. The addition of a methoxy group to the ethyl chain of theobromine reduced the diuretic potency. The same appeared true when a methyl group was attached to the allyl radical.

TABLE 3

Respiratory stimulation by theobromine derivatives when given intravenously to dogs deeply anesthetized with sodium phenobarbital

COMPOUND	NUMBER OF ANIMALS USED	DOSE	AVERAGE PER CENT INCREASE OF VOLUME OF EXPIRED AIR FOR 30 MINUTES FOLLOWING INJECTION
		mg. per kg.	
Caffeine	6	10	12.6
Ethyl Theobromine	6	10	34.7
Propyl Theobromine	5	10	82.5
Butyl Theobromine	3	10	41.2
*Iso*amyl Theobromine	3	10	37.3
Allyl Theobromine	3	10	25.1
Methallyl Theobromine	3	10	50.8
Crotyl Theobromine	4	10	60.3
Methoxyethyl Theobromine	3	10	7.2
Lobeline Sulfate	5	0.25–0.5	39.2
Nikethamide	4	20–40	11.2

Tests on *trained, unanesthetized dogs* were made according to the procedure of a previous communication (1). All drugs were injected intravenously, the dose in each case being 10 mg. per kg. Total urine output was collected for 90 minutes after injection of the test substance. In control experiments, 1 cc. per kg. of 0.9 per cent sodium chloride solution was given by vein, since solutions of the compounds were made in this volume also. The results are found in table 4. Both the ethyl and propyl theobromines were more potent than caffeine. The order of effectiveness was propyl > ethyl > methyl (caffeine) > methallyl > butyl > allyl > methoxyethyl > *iso*amyl. The crotyl derivative appears to have a suppressive effect on the urine output. Addition of a methoxy radical to the ethyl group reduces the diuretic action, while a methyl group attached to an allyl chain seems to increase the action of the latter.

Some of the compounds produced nausea (evidenced by salivation) and vomiting. The crotyl compound caused vomiting in 4 of 5 dogs, the methallyl in 3 of 6, the butyl in 2 of 7, and the *iso*amyl in 1 of 7 animals. Vomiting commenced from 3 to 5 minutes after injection of the substances, the retching movements continuing usually for 2 to 5 minutes.

TABLE 4

Diuretic action of theobromine derivatives in trained unanesthetized dogs

THEOBROMINE DERIVATIVE*	NUMBER OF DOGS USED	AVERAGE URINE OUTPUT FOR 90 MINUTES	NUMBER OF DOGS WHICH VOMITED
		cc.	
Caffeine	10	94.0	0
Ethyl†	6	114.0	0
Propyl	6	117.3	0
Butyl	7	83.6	2
*Iso*amyl	7	74.6	1
Allyl	6	80.7	0
Methallyl	6	85.9	3
Crotyl	5	40.6	4
Methoxyethyl	4	74.8	0
Theobromine	8	53.4	0
0.9% NaCl Sol.	9	61.5	0

* All doses were 10 mg. per kg., injected intravenously, usually as a 1% solution in 0.9% NaCl solution.

† Previously reported (1).

Action in man. Tests of diuretic action of allyl theobromine, theophylline, and caffeine were made on 6 normal adult *human beings.* The procedure has been previously described (1). The drugs were given by mouth in gelatin capsules at 8:00 a.m., the dose being 200 mg. in each case. Subjects did not know what compound they received. In order to avoid any bias, the order of administration of the test substances was different for each subject. Daily urine output was measured from 8:00 a.m. to 4:00 p.m. for a total of 9 days. The subjects continued their usual work around the laboratories.

The differences in results between the 3 substances, which are not detailed here, were not significant, although theophylline seemed to be slightly stronger than the other 2 compounds. In another test, propyl theobromine was directly compared with caffeine. The urine output following the former was greater than that following the latter.

The critical fusion frequency and the finger-tapping rate were determined in the same subjects according to the methods of Simonson and Enzer (10, 11). These were measures of the efficiency of visual centers, and the activity of motor centers, respectively.

The differences in results between compounds were so slight that they were inconclusive. There was a suggestion that caffeine and propyl theobromine were more powerful in increasing the finger-tapping rate. The final conclusion must await further study with a much larger group of subjects.

Untoward symptoms occurred. Propyl theobromine produced nausea in 73 per cent of the trials; the allyl derivative, 50 per cent; theophylline, only once; and caffeine, not at all. Nausea appeared about 1 hour after ingestion of the capsule and lasted 1 to 2 hours. Both caffeine and allyl theobromine were followed by a tired feeling the next morning more frequently than theophylline. Allyl and propyl theobromines produced a jittery sensation in 7 of 18 and 7 of 15 trials, respectively, while it was noted only twice with theophylline, and none with caffeine.

In addition to nausea following ingestion of propyl theobromine, vomiting, hiccoughs, and sweating occurred 3, 2, and 5 times, respectively. Allyl theobromine also caused vomiting on one occasion. In another trial, a dose of 100 mg. of the propyl derivative given orally to 3 individuals produced sweating and nervousness in all 3 persons, and nausea in 2 of the subjects.

SUMMARY

A series of 1-substituted theobromine derivatives has been investigated for some of their pharmacologic actions with the following results:

1. From the median lethal doses by intravenous injection in mice, the compounds showed the following order of decreasing toxicity: allyl > ethyl > crotyl > methyl (caffeine) > propyl > butyl > *iso*amyl > methallyl > methoxyethyl. The median convulsive doses revealed the same order except that the butyl and *iso*amyl derivatives were not convulsants.
2. Caffeine, allyl theobromine, and propyl theobromine inhibited weight gain of young rats when fed for 28 days in percentages of 0.3 and 0.5 in the food. Smaller amounts did not have any effect. Allyl theobromine inhibited growth of young rats to a much greater extent than the other 2 substances.
3. Caffeine was more stimulating to motor activity of rats in spring-suspended cages than the theobromine compounds which have the same action. By the same procedure, theophylline was shown to be stronger than caffeine, but much less effective than *d*-amphetamine sulfate.
4. Propyl theobromine produced much greater respiratory stimulation than any other member of this series, when given to dogs deeply anesthetized with phenobarbital sodium. Compared with caffeine, nikethamide, and lobeline,

the propyl derivative was more than 6 times as effective as the first 2 compounds and twice as active as lobeline.

5. In tests of diuretic action, rats showed greater urine outputs with caffeine than with 1-substituted theobromines. In dogs, the diuretic potency had the following order: propyl > ethyl > methyl (caffeine) > methallyl > butyl > allyl > methoxyethyl > *iso*amyl. The crotyl derivative had an inhibiting effect on the urine output.

6. Allyl theobromine, propyl theobromine, caffeine, and theophylline were tested in a few human subjects in a dose of 200 mg. given orally. There was evidence that propyl theobromine was more powerful in increasing the urine output than caffeine; and that the latter, in turn, was slightly more powerful than the other two products. Untoward symptoms were observed—more with allyl and propyl theobromines.

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THE EFFECTS OF THIOURACIL ON THE HEMATOPOIETIC SYSTEM OF THE ALBINO RAT[1]

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Thiourea and thiouracil cause enlargement, hyperemia and hyperplasia of the thyroid gland of albino rats. Concomitantly a state of hypothyroidism is indicated by a decrease in food intake, growth, development and oxygen consumption (1), (2), (3), (5). Both drugs exert a thyroid-depressant action in human beings suffering from thyrotoxicosis and hyperthyroidism (4), (5). Observations on the clinical use of these drugs include numerous references to toxic reactions. Disturbances of the hematopoietic system, such as leucopenia and agranulocytosis, have caused the greatest concern (lit. regarding thiouracil is cited in refs. 6 and 7). Eleven cases of agranulocytosis due to thiouracil have been reported (5), (7), (8), (9), (10) (11), (12), (13), (14), of which five have terminated fatally (8), (9), (10), (13), (14).

The production and prevention of granulocytopenia in rats fed thiourea (15) has been discussed and the effects of thiouracil on the peripheral blood of rats has been noted (16). Although the incidence of fatality due to agranulocytosis following thiouracil therapy is not high it is believed the severe nature of the reaction justifies further study of the hematopoietic system as affected by this drug. The present investigation is concerned with the effects of measured daily doses on the peripheral blood and bone marrow of albino rats.

MATERIALS AND METHODS. Thirty-eight female and fifteen male albino rats of the Rockland Farms strain, 8 months of age, were selected for the experiment. They were kept in metabolism cages, at a unfirom temperature, were fed a standard diet of Rockland Farms' Vit. D-free rat pellets[4] and had free access to fresh water. All the animals had been handled daily since birth, to eliminate factors which might influence the procuring of representative experimental data. One group of 6 rats served as control. The remainder were fed graded doses of a suspension of thiouracil in water daily, Sundays and holidays excluded. The intubation method devised by Lehr (17) was used, to assure a constancy of dosage. The drug was administered at the same hour each day and all observations were made at 24 hour intervals with a constant relation to the feeding of the drug. The animals were weighed weekly.

Twenty-seven rats were selected for study of the effects of thiouracil on the total and differential white blood cell counts. Prior to initiation of therapy, "normal" values were obtained for each animal. The rats received daily doses of either 0.05, 0.1, 0.2 or 0.225 gm.

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[4] All brood animals of the Rockland Farms have been satisfactorily maintanined on this diet since 1935. For the purposes of our experiments, therefore, it seemed unwise to alter the diet to which this strain of rats had been accustomed for many generations.

of the drug for varying periods of time. Total and differential white blood cell counts were performed on each animal daily (table 1).

Twenty rats were selected for the purpose of studying the effects of thiouracil on the bone marrow, as well as on the peripheral blood. They were fed daily doses of 0.1 or 0.2 gm. of of the drug for varying periods of time (table 3).

Complete blood counts were performed on 3 control rats and on 18 treated rats prior to the initiation of therapy and at weekly intervals thereafter. Blood for all counts was obtained by snipping the tail. In all, 176 total and differential white blood cell counts were made on untreated and 361 on treated animals. Twenty-seven complete blood counts were made on untreated and 45 on treated animals.

All the rats, except those of Group 9 and 3 of those in Group 10, which died during the course of the experiments, were sacrificed one day after receiving their last dose of the drug. Complete autopsies were performed. The thyroid gland was dissected away from the surrounding structures, weighed on an analytical balance and the ratio of thyroid weight to body weight computed. Sections were taken from all the organs, fixed in Zenker's or formalin solution and stained routinely with hematoxylin and eosin. In addition, sections from femoral bone marrow of every animal were treated with the Wohlbach modification of the Giemsa stain. Multiple smears of femoral bone marrow were obtained from 17 rats in accordance with a recently described technique (18), and treated with the Jenner-Giemsa stain.

RESULTS AND DISCUSSION. (a) *Hemogram of the normal rat.* It is generally acknowledged that each element of the blood of the normal rat shows a wider range of variation than the same element in normal human blood. The range of red blood cells has been given as 7 to 10 million per cu. mm. (15), (19), (20). Repeated erythrocyte counts made in this laboratory on 21 rats varied from 5.05 to 9.31 million per cu. mm. Determinations of hemoglobin have been reported as ranging from 10 to 23.3 gms. per 100 cc. of blood (15), (16), (20), (21). Determinations made on 21 rats in this laboratory ranged from 13.5 to 17.5 gms. per 100 cc. of blood. The range of white blood cells has been given as 6 to 27.1 thousand per cu. mm. of blood (15), (16), (19), (21), and the ranges for individual types of leucocytes have been reported in percentage as polymorphonuclear neutrophils, 15 to 47; lymphocytes, 47 to 87; monocytes, 0 to 7; eosinophils, 0 to 7 and basophils, 0 to 1 (15), (19), (20). The range of white blood cells as determined in this laboratory on 53 rats was 4.35[5] to 26.25 thousand per cu. mm. of blood and of individual types of leucocytes, 6 to 57 per cent were polymorphonuclear neutrophils, 38 to 88 per cent were lymphocytes, 0 to 7 per cent monocytes and 0 to 7 per cent eosinophils (tables 1, 2, 3).

(b) *Hemogram of the rat following treatment with thiouracil.* Under treatment with thiouracil 8 of 15 rats showed a slight decrease in the number of red blood cells per cu. mm. and the count of 7 rats remained approximately stationary, simulating that of the rats in the control group. Ten of the 15 animals showed a definite decrease in the hemoglobin content of the blood, 4 showed a slight decrease and 1 remained stationary (table 2). This confirms a previous observation (16). Similar findings have been reported for both rats (22), (23) and human beings treated with sulfonamides (24).

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[5] This represents a single determination which was outside the figures previously reported by other investigators. All other determinations fell within a range of 6.40 to 26.25 thousand white blood cells per cu. mm. of blood.

TABLE 1

General data from rats before and after treatment with thiouracil

GROUP NO.*	THIOURACIL			PER CENT WEIGHT CHANGE	TW/BW†	TOTAL NUMBER COUNTS	LEUCOCYTES		DIFFERENTIAL WHITE COUNT (PER CENT)							
	Amount								Polys		Lymph.		Eosino.		Mono.	
	Daily	Total	Total				Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range
	gm.	*gm.*	*days*				*Th./cmm.*	*Th./cmm.*								
Control			30	+0.2	10	18	16.25	8.35–26.95	27	11–57	67	38–85	4	0–12	2	0–4
1																
before						15	15.10	7.85–24.00	32	20–44	61	54–71	4	1–9	3	1–4
after	0.05	0.25	5	+3.4	12	15	20.36	2.70–28.10	28	11–42	67	50–85	2	0–7	2	0–5
2																
before						15	10.40	7.95–12.30	27	19–37	66	53–76	5	3–6	3	1–5
after	0.05	0.50	10	+2.1	17	30	13.50	7.70–27.40	23	9–38	71	50–85	3	1–6	4	0–7
3																
before						15	13.00	6.50–26.25	28	15–41	67	53–83	2	0–4	3	0–5
after	0.05	1.00	26	−2.2	16	60	11.45	3.70–22.70	24	8–49	70	46–91	3	0–9	3	0–5
4																
before						15	12.50	7.75–20.85	13	7–22	79	68–88	6	2–10	2	0–5
after	0.1	0.50	5	+0.8	11	15	11.20	8.20–15.00	17	2–29	77	65–96	3	1–7	3	1–5
5																
before						15	10.30	6.95–14.75	25	14–34	65	50–76	7	2–16	3	1–7
after	0.1	1.00	12	−1.4	20	30	14.80	6.30–22.80	25	10–41	68	52–85	4	0–10	3	0–8
6																
before						15	13.55	7.50–24.75	14	9–39	72	52–85	3	1–6	3	2–6
after	0.1	2.00	25		18	60	11.20	5.15–20.65	16	7–32	74	53–88	4	0–15	2	0–5
7																
before						15	9.40	6.40–17.30	15	6–21	78	71–86	4	2–6	3	1–4
after	0.2	1.00	5	−3.1	16	15	9.20	6.70–14.75	27	8–52	65	43–90	5	0–12	3	0–7
8																
before						15	14.40	9.50–18.50	25	10–48	69	47–85	3	0–8	3	1–5
after	0.2	4.00	28	−3.1	24	60	13.50	6.15–25.50	20	4–65	75	30–94	3	0–9	2	0–5
9																
before‡						15	11.80	4.35–18.70	30	19–39	60	51–72	5	2–10	5	2–6
after	0.225	2.00	10	−18.4	18	27	14.50	7.85–24.00	43	21–74	47	23–66	5	1–9	5	1–6

* *3 rats per group.*

† TW/BW—thyroid weight/body weight—expressed in whole numbers actually representing the 4th and 5th decimal places.

‡ Rats died on 7th, 9th and 11th day of treatment, respectively.

The range and the average figures for the total white blood cell counts and the percentage distribution of the various types of leucocytes have been tabulated for each group of animals (table 1). The elevation of the polymorphonuclear neutrophilis noted in several subjects was probably due to kidney block which is mentioned below under post-mortem findings.

Changes from time to time in the total and differential white blood cell counts of the individual untreated animal were slight, but variations from animal to animal were large. The corresponding values obtained for each of the treated animals showed considerable fluctuation.

TABLE 2

Influence of thiouracil on the hemoglobin and erythrocyte counts of 15 rats before and after treatment with the drug

GROUP	THIOURACIL		TOTAL DAYS	HEMOGLOBIN		ERYTHROCYTES	
	Daily	Total		Initial	Final	Initial	Final
	gm.	*gm.*		*gm./100 cc.*		*mill./cu. mm.*	
Control			50	13.75 14.50 14.75	13.00 14.00 14.50	8.02 7.56 8.51	7.71 9.00 7.88
5	0.1 0.1 0.1	1.0 1.0 1.0	12 12 12	16.50 16.50 15.50	12.25 11.50 11.00	6.78 5.66 5.05	5.22 5.00 5.06
9	0.225 0.225 0.225	2.25 1.575 1.80	12 8 10	17.50 15.00 17.50	13.50 12.50 12.50	6.19 6.48 6.80	5.08 6.31 5.54
10	0.1 0.1 0.1 0.1 0.2 0.2 0.2 0.2 0.2	6.0 6.0 6.0 6.0 4.0 4.0 4.0 4.0 4.0	78 78 78 78 24 24 24 24 24	17.00 15.50 14.00 14.00 14.75 13.50 15.00 13.00 15.00	14.00 12.00 12.50 12.00 13.25 12.00 13.00 12.75 14.00	7.64 6.99 7.03 8.09 6.93 8.03 9.31 7.28 8.19	6.55 6.36 5.94 6.55 8.36 8.32 8.43 7.04 6.52

In view of the above it is difficult to draw conclusions concerning the effects of thiouracil upon the hemogram. However, within the limits of our experiments in rats, two things seem clear: 1) thiouracil is capable of producing a mild anemia and 2) it does not produce a *continuing, progressive* leucopenia or granulocytopenia.

(c) *The bone marrow in rats treated with thiouracil.* In a previous experiment myelograms were made for each of a group of 12 normal albino rats (18). The range of figures thus obtained was used as a basis for comparison with the bone marrow counts made in the present study. The individual cell types did not vary qualitatively from the normal. Quantitatively, 9 rats exhibited a decided increase in the erythroid and a slight to to moderate decrease in the more immature forms of the myeloid elements (tables 4, 5). This finding was apparently related to the total amount of the drug given and the period of time over which it was administered.

It is concluded that, in the rat, the bone marrow may be a more reliable index of the effects of thiouracil on the hematopoietic system than the peripheral blood.

TABLE 3

Data from individual rats, in which bone marrow studies were done, after treatment with thiouracil

RAT NO.	THIOURACIL		TREATMENT	WEIGHT	TW/BW*	LEUCOCYTES									
	Daily	Total		(% change)		Total		Differential Count (per cent)							
						Init.	Fin.	Polys		Lymph.		Eosino.		Mono.	
								Init.	Fin.	Init.	Fin.	Init.	Fin.	Init.	Fin.
	gm.	*gm.*	*days*			*cu. mm.*									
a	0.1	1.0	12	−9.0	14	16.90	16.20	22	21	74	72	3	4	1	2
b	0.1	1.0	12	−4.2	11	14.75	16.90	35	24	57	69	8	7	0	0
c	0.1	1.0	12	−2.7	12	27.80	22.00	10	19	89	79	5	1	0	1
d	0.1	1.0	12	+2.5	19	9.50	15.45	29	34	66	59	5	5	0	2
e	0.1	6.0	78	+11.2	24	12.85	7.95	24	16	74	76	1	6	1	2
f	0.1	6.0	78	+19.3	22	16.80	10.15	21	19	76	75	1	2	2	4
g	0.1	6.0	78	+18.0	21	13.10	9.15	26	17	70	82	4	1	0	0
h	0.1	6.0	78	+3.2	19	14.40	5.05	22	27	73	69	3	2	2	2
i	0.2	2.0	11	−4.5	14	12.25	10.05	22	18	76	79	1	3	1	0
j	0.2	2.0	11	−19.7	18	15.75	13.20	26	43	71	55	2	2	1	0
k	0.2	4.0	24	−3.9	13	17.75	10.95	29	17	66	80	5	2	0	1
l	0.2	4.0	24	−7.6	17	13.40	15.75	38	36	58	58	4	6	0	0
m	0.2	4.0	24	−6.2	15	9.60	6.65	21	22	79	78	0	0	0	0
n	0.2	4.0	24	−10.5	22	8.05	6.05	35	18	62	81	2	0	1	1
p	0.2	4.0	24	+15.9	16	15.10	15.10	25	16	74	77	1	4	0	3
q	0.2	4.0	24	−16.6	20	9.45	9.10	24	51	76	48	0	0	0	1
r	0.2	4.0	24	−35.3	21	10.25	8.85	27	44	69	55	2	0	2	1

* TW/BW—ratio of thyroid weight to body weight expressed in whole numbers actually representing the 4th and 5th decimal places.

(d) *The toxic dose of thiouracil.* With the exception of 3 animals in Group 10, all rats receiving 0.2 gm., or less, of thiouracil daily survived the specified test periods. The 3 rats of Group 9 succumbed following 12 daily doses of 0.225 gm. of the drug. In the course of other experiments, it has been found that thiouracil in 0.3 gm. doses daily was fatal in three days. It is therefore concluded that, under the conditions noted, the sub-acute lethal dose lies between 0.2 and 0.225 gm. the drug daily, and that the L.D. 50 lies between 0.6 and 0.9 gm. Dosages slightly less than this have been used by Astwood (25) as a 1% mixture in the food. One third of the animals so treated developed urinary concretions from which some (percentage not stated) succumbed. However, those that survived were able to continue the regime for as much as 5 months without "symptoms

TABLE 4

Study of the various types of cells in the bone marrow of the albino rat with and without thiouracil therapy. Figures in each column represent the number of cells found per 1000 cells counted. The average myeloid/erythroid ratio in untreated rats was 1.78

RAT NO	Control group range	a	b	c	d	e	f	g	h	i	j	k	l	m	n	p	q	r
Thiouracil No. of days used	0	12	12	12	12	78	78	78	78	11	11	24	24	24	24	24	24	24
Total dose (gm.)	0	1.0	1.0	1.0	1.0	6.0	6.0	6.0	6.0	2.0	2.0	4.0	4.0	4.0	4.0	4,0	4.0	4.0
Mature neutrophils	168–281	226	224	235	199	215	160	184	180	217	288	169	206	223	207	263	238	203
Immature neutrophils	61–199	170	85	78	99	82	80	79	86	81	131	91	79	18	17	51	90	61
Metamyelocytes neutrophils	17–41	35	31	14	10	38	25	18	17	14	35	25	15	24	18	33	31	53
"Ring cells"	40–88	66	43	64	60	10	44	35	62	24	16	43	30	32	48	39	32	53
Myelocytes neutrophils	12–36	35	18	11	15	27	12	15	19	64	22	14	17	21	16	20	10	21
Promyelocytes neutrophils	0–8	6	6	4	4	1	0	0	0	8	7	4	2	0	0	2	1	0
Mature eosinophils	24–61	44	100	64	78	81	45	55	19	31	80	81	32	42	56	103	32	39
Immature eosinophils	12–60	41	42	24	46	27	34	22	29	20	26	21	24	26	21	18	23	10
Metamyelocytes eosinophils	5–18	12	42	21	29	10	6	7	6	8	15	13	9	4	6	15	4	7
Myelocytes eosinophils	1–4	3	8	2	8	6	4	2	3	1	5	1	2	2	1	7	2	1
Basophils	0–2	1	1	0	0	0	0	0	0	3	0	1	0	1	0	0	0	0
"Blast cells"	23–61	34	36	47	48	42	35	25	45	38	35	33	31	7	22	30	10	7
Erythroblasts	66–133	54	102	122	113	114	169	99	132	112	72	122	112	246	168	191	203	178
Normoblasts	175–272	194	139	196	172	216	318	373	282	276	154	302	343	278	357	184	266	294
Lymphocytes	17–52	27	20	30	32	16	15	22	13	24	36	18	23	11	3	6	14	8
Monocytes	0–6	1	1	3	5	1	0	0	0	2	4	0	3	0	1	0	2	1
Megakaryocytes	0–6	1	0	6	3	4	0	5	2	2	4	0	2	3	4	1	0	5
Macrophages	0–4	2	1	2	2	1	2	1	0	1	2	1	0	2	1	1	1	1
"Kugelhaufen"	0–2	0	3	0	2	1	5	1	1	0	0	2	2	2	4	5	2	0
Plasma cells	4–10	8	9	3	3	5	10	8	5	9	3	3	11	1	7	2	3	3
Hemocytoblasts	1–9	3	9	11	4	4	5	3	3	4	1	2	1	1	4	1	3	1
Degenerated cells	35–87	38	79	60	73	73	30	45	46	31	34	54	56	26	9	28	12	24
Myeloid/erythroid ratio	1.33–2.66	2.58	2.50	1.62	1.91	1.59	0.84	0.88	0.95	1.39	2.50	1.09	0.91	0.82	0.84	1.55	1.0	1.03

or signs other than those attributable to a state of hypothyroidism." When these statements are considered in relation to our own data it is clear that rapidly attained blood levels predispose to renal complications which may result fatally, although the total dosage received may be far below that tolerated when the drug is so administered as to promote slower absorption.

(e) *Autopsy findings in rats treated with thiouracil.* At autopsy, enlargement and hyperemia of the thyroid gland was observed in all cases. The ratio of thyroid weight to body weight, in the majority of instances, increased in direct relation to the amount of drug administered daily and the length of time over which continued.

The presence of a small amount of fine, yellowish-white granular material was noted in the renal pelvices of one rat in Group 5, 2 rats in Group 10 and in the 2 rats receiving 0.3 gm. thiouracil daily. The kidneys were soft, swollen and enlarged. In the latter two instances the granular material was visible in the medullary tissues, the pelvices and the ureters. The ureters were dilated and the urinary bladder was empty.

TABLE 5

Comparison of myeloid/erythroid ratios in bone marrow of normal rats and rats treated with thiouracil

MYELOID CELLS	RANGE	AV.*	RANGE	AV.*	RANGE	AV.*
	12 normal rats		4 rats 0.1 gm. daily (78 days)		7 rats 0.2 gm daily (24 days)	
Mature granulocytes .	192–347	283	205–296	242	201–367	271
Immature granulocytes .	73–259	153	102–120	110	65–131	91
Metamyelocytes	22–59	39	24–57	39	19–68	35
"Ring" cells .	40–88	54	35–62	45	30–53	40
Myelocytes	13–40	25	14–33	22	11–28	19
Promyelocytes	0–8	3	0–1	0	0–4	1
"Blast" cells	23–61	36	25–45	38	7–33	20
ERYTHROID CELLS						
Normoblasts	175–272	216	216–373	272	184–357	289
Erythroblasts	66–133	80	99–169	129	112–246	174
Myeloid/Erythroid . .	1.33–2.66	1.78	0.84–1.59	1.07	0.82–1.55	1.03

* Representing the nearest whole number.

One rat in Group 10 lost hair from the ventral thorax and abdomen during the course of the experiment. At autopsy a moderate amount of clear, pale, yellow fluid was present in her pleural and abdominal cavities.

Microscopic examination of post-mortem sections from all organs of each animal revealed nothing of pathological significance except in the bone marrow, thyroid gland and the kidneys. Variations in the bone marrow have already been described. In the thyroid varying degrees of hyperplasia and diminution in colloid were noted in all animals. In those kidneys showing precipitated thiouracil on gross examination, cloudy swelling of the tubular epithelium with desquamation, capillary dilatation and engorgement, and a slight to moderate degree of tubular dilation were observed. The bone marrow appeared to be hyperplastic in every rat.

A total of 8 rats died spontaneously during the course of the experiment while receiving relatively high doses of thiouracil. Oliguria was present in each but anuria was not observed. The weight loss of individual subjects was considerable. Terminal white blood cell counts performed on each of these rats showed a high percentage of polymorphonuclear neutrophils (tables 1, 3). At autopsy the drug was present in the kidneys of 6 of the 8 rats. Apparently the cause of death was a renal block with associated uremia, although this was not proven. Precipitation of the drug in the kidneys may have resulted from the high blood and renal concentration reached following a single large daily dose of thiouracil.

SUMMARY

1) Forty-seven albino rats were fed thiouracil in doses ranging from 0.05 to 0.225 gm. daily for from 5 to 78 days.

2) A slight decrease in the total number of red blood cells and a slight to moderate decrease in the hemoglobin content of the peripheral blood was noted in a high percentage of the rats upon which complete blood counts were performed. In a study of 361 specimens taken from treated animals no *sustained* variations from the normal range of total and differential white blood cell counts were noted.

3) Smears of the bone marrow revealed a definite decrease in the myeloid-erythroid ration in 3 of 4 animals receiving 0.1 gm. of thiouracil daily for 78 days and in 6 of 7 animals to which 0.2 gm. of drug was given daily for 24 days.

4) The presence of granular deposits in the kidney pelvices was noted at autopsy in 7 rats.

5) In addition to the changes in the bone marrow, microscopic examination of sections of organs taken at autopsy revealed alterations only in the thyroid gland and in the kidneys. The former showed varying degrees of hyperplasia and diminution of colloid; the latter exhibited some evidences of tubular damage.

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THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND SPASMOLYTIC ACTION OF SOME NEW ANTICOLINERGIC ESTERS

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The pharmacological literature during recent years has contained numerous reports describing the chemical structure and biological action of many new synthetic substances developed in an effort to replace atropine as an antispasmodic agent. This literature has been extensively reviewed recently by Blicke (1) and need not be repeated here. An examination of the data reported indicates that most of these substances are distinctly less spasmolytic than atropine and that side-reactions can be elicited with some of them when they are administered in relatively large amounts. Nechles et al. (2) reported aboliton of the vasodepressor action of acetylcholine in dogs after large doses of β-diethylaminoethyl diphenylacetate (Trasentine). Meier and Hoffmann (3) report mydriasis, inhibition of salivary secretion and the antagonizing of the action of acetylcholine on the frog heart by both β-diethylaminoethyl diphenylacetate and β-diethylaminoethyl phenylcyclohexaneacetate (Trasentine-H). Similarly, actions on parasympathetically innervated structures other than the intestine have been reported for γ-diethylamino-β,β-dimethylpropyl tropate (Syntropan) and related structures by Fromherz (4).

This laboratory has determined the spasmolytic action of a number of substituted acetic acid esters of aminoalcohols and the results obtained with a few of these have been reported by Lands and Nash (5) and Lands, Nash and Hooper (6). The series of compounds described in tables 1 and 2 have been made available to us for pharmacological investigation. The synthesis of the basic esters of p-xenylacetic acid has been described by Blicke and Grier (7), of naphthylacetic acid by Blicke and Feldkamp (8), and of α-thienylacetic acid by Blicke and Tsao (9). The phenyl- and cyclohexylacetic acid esters (table 1, Nos. 3, 5, 6 and 7) are known compounds. They are included here for comparison. In this communication we have described the results obtained with several new esters of α-thienylacetic acid, and α-thienylglycolic acid. Intestinal spasmolytic action and anti-cholingeric action in various other parasympathetically innervated structures has been determined.

RESULTS. 1. *Action on the Small Intestine.* a. Effect of the Acid Portion of the Ester. Isolated segments of the small intestine of the rabbit were suspended in Sollmann-Rademaker solution and the action of drugs determined by the method of Magnus. Results obtained are shown in table 1. The acetic and propionic acid esters of β-diethylaminoethanol induce contractures of the intestinal segment that are indistinguishable from those caused by acetylcholine although much higher concentrations of the former compounds are required.

This action is inherent in the basic alcohol since similar stimulation can be induced by the alcohol alone, when present in relatively high concentration. Esterification with acetic or propionic acids thus increases this inherent cholinergic action (Lands et al., 6). The stimulant action of the ester is abolished by the replacement of one of the hydrogen atoms of the acetate by an hydroxyl group, as in β-diethylaminoethyl glycolate. The substitution of a phenyl group for one hydrogen atom of the acetate results in an ester that is definitely spasmolytic. The addition of an hydroxyl to the latter to form β-diethylaminoethyl phenylglycolate gives a compound that is more spasmolytic than either of the previously mentioned mono-substituted acids. Saturation of the phenyl group to produce the corresponding cyclohexyl analogs (table 1, Nos. 5 and 6) results in a further increase in spasmolytic action. The replacement of two hydrogen atoms by phenyl groups as in β-diethylaminoethyl diphenylacetate (Trasentine) further increases spasmolytic activity. Meier and Hoffmann (3) investigated the phar-

TABLE 1

Spasmolytic action of substituted acetic acid esters of β-diethylaminoethanol

COMP. NO.		ACTION ON THE ISOLATED RABBIT JEJUNUM (MAGNUS)	
		Acetylcholine[*]	Barium Chloride[*]
		1-	1-
1.	Acetic[§]	Stimulates	
2.	Glycolic[†]	10,000-20,000	
3.	Phenylacetic	100,000-200,000	10,000-20,000
4.	Phenylglycolic[†]	200,000-500,000	
5.	Cyclohexaneacetic	200,000-500,000	20,000-40,000
6.	Cyclohexaneglycolic	500,000	10,000-20,000
7.	*Diphenylacetic*[‡]	*500,000-M(illion)*	200,000
8.	Phenyl-α-thienylacetic	2-5M	200,000-400,000
9.	Di-α-thienylacetic	1-4M	200,000
10.	Diphenylglycolic	40-50M	100,000-200,000
11.	Phenyl-α-thienylglycolic	40-80M	200,000-400,000
12.	Cyclohexyl-α-thienylglycolic	60-100M	200,000-500,000
13.	p-Xenyl-α-thienylacetic	500,000	500,000
14.	p-Xenyl-α-thienylglycolic	500,000	500,000
15.	α-Naphthylacetic	50,000-200,000	50,000-200,000
16.	p-Xenylacetic	50,000-200,000	50,000-200,000

[*] Acetylcholine chloride was added directly to the bath to make a final dilution of 1-1M to 1-2M.

Barium chloride was added directly to the bath to make a final dilution of 1-10,000 to 1-20,000.

[†] These compounds were supplied to us as oily hydrochlorides without definite identifying physical constants. Inasmuch as their physiological activity falls in line in the series, they are included for comparison.

[‡] Trasentine. Meischer and Hoffmann, U. S. 2,079,962 (1937). Cf. Meier and Hoffmann (3).

[§] All esters were used as the hydrochloride salt in aqueous solution, except as noted above.

macological action of β-diethylaminoethyl phenylcyclohexaneacetate hydrochloride (Trasentine-H) and dicyclohexaneacetate hydrochloride and found the former to be definitely more spasmolytic than Trasentine and the latter to be less spasmolytic. This suggests that asymmetry of the acetate favors spasmolytic action. In agreement with this, it was found that β-diethylaminoethyl phenyl-α-thienylacetate is more spasmolytic than the corresponding diphenylacetate or di-α-thienylacetate. The replacement of the remaining hydrogen atom of the acetate by an hydroxyl causes a further marked increase in spasmolytic potency. Lehmann and Knoefel (10) have reported β-diethylaminoethyl phenylcyclohexaneglycolate to be about ten times more spasmolytic than Trasentine. Similarly, we have found the phenyl-α-thienylglycolate ester to be

TABLE 2

Spasmolytic action of substituted acetic acid esters of several aminoalcohols

COMP. NO.	ACID	ALCOHOL	ACTION ON THE ISOLATED JEJUNUM (MAGNUS)	
			Acetylcholine*	Barium Chloride†
			1–	1–
8	Phenyl-α-thienylacetic‡	β-Diethylaminoethanol	2–5M(illion)	200,000
28	Phenyl-α-thienylacetic	Piperidinoethanol Methylbromide	2–4M	200,000
29	Phenyl-α-thienylacetic	γ-Diethylaminopropanol	2–4M	200,000
30	Phenyl-α-thienylacetic	β-Dimethylaminoethanol	500,000–1M	150,000
31	Phenyl-α-thienylacetic	γ-Dimethylamino-β, β-dimethylpropanol	3–5M	100,000
11	Phenyl-α-thienylglycolic	β-Diethylaminoethanol	40–80M	200,000
32	Phenyl-α-thienylglycolic	β-Piperidinoethanol	20–40M	400,000
33	Phenyl-α-thienylglycolic	γ-Diethylaminopropanol	30–50M	200,000
34	Phenyl-α-thienylglycolic	β-Dimethylaminoethanol	30–50M	200,000
35	Phenyl-α-thienylglycolic	γ-Dimethylamino-β,-β-dimethylpropanol	75–100M	100,000
36	Phenyl-α-thienylglycolic	β-Dibutylaminoethanol	200,000–400,000	200,000–400,000
12	Cyclohexyl-α-thienylglycolic	β-Diethylaminoethanol	60–100M	200,000–500,000
37	Cyclohexyl-α-thienylglycolic	β-Piperidinoethanol	60–100M	200,000–500,000
38	Cyclohexyl-α-thienylglycolic	γ-Diethylaminopropanol	60–100M	200,000–1M

* Acetylcholine chloride, 1–1M or 1–2M.

† Barium Chloride, 1–10,000 or 20,000.

‡ All esters were used as the hydrochloride salt in aqueous solution.

ten to fifteen times more spasmolytic than the corresponding acetate (5). The saturation of the phenyl ring of this compound to form cyclohexyl-α-thienylglycolate causes a very great increase in spasmolytic activity.

A few experiments were carried out with compounds wherein the phenyl was replaced by p-xenyl and α-naphthyl groups. Poor solubility in neutral solution made determination of results difficult. However, these substances appeared to have little or no anticholingeric action. Relaxation was primarily due to a direct action on the muscle cell (table 1, Nos. 13–16). We believe these experiments indicate a loss of anticholinergic action when p-xenyl or α-naphthyl groups are substituted for phenyl or α-thienyl structures.

b. Effect of the Aminoalcohol Portion of the Ester. It was previously noted that choline-like action is found with β-dimethyl- and β-diethylaminoethanol, γ-diethylaminopropanol and β-piperidinoethanol. The alcohols wherein the substituents on the nitrogen are larger than ethyl were found to be papaverine-like in action.

Esters of sevral different aminoalcohols with the same acids were available to us and a determination of the effects of structural modification of the aminoalcohol on spasmolytic action was thereby possible. Results obtained on the isolated intestinal segment are shown in table 2. Examination of the data indicates that when the substituents on the nitrogen are dimethyl or diethyl the resultant ester is anticholingeric. Increase in the size of these groups to propyl (Meier and Hoffmann 3), butyl or larger groups results in an ester that appears to be only papaverine-like in action. The group between the nitrogen and the carboxyl may be ethyl or propyl and may contain methyl substitutions, as in compound Nos. 30 and 34, and still exert and anticholingeric spasmolytic action. Our series did not include esters of alcohols beyond aminopropanol.

Various aminoalkyl esters of phenyl-α-thienylacetic, phenyl-α-thienylglycolic and cyclohexyl-α-thienylglycolic acids were made available to us for pharmacologic investigation. It will be noted (table 2) that the activity of these esters is determined largely by the structure of the substituted acetic acids. The phenyl-α-thienylglycolates are distinctly more spasmolytic than the corresponding phenyl-α-thienylacetates, with the exception of the ester of β-dibutylaminoethanol. This latter compound is a weak spasmolytic drug and appears to be papaverine-like in action inasmuch as essentially the same values are obtained against both acetylcholine and barium induced contratures. The cyclohexyl-α-thienylglycolates are most spasmolytic. Anticholinergic (spasmolytic) action appears to be greatest for the esters of the alcohols γ-dimethylamino-β,β-dimethylpropanol and β-diethylaminoethanol and least for β-dimethylaminoethanol. The papaverine-like action against barium chloride induced contractures is of the same order of magnitude for all of these substances although it would appear that the esters of γ-dimethylamino-β,β-dimethylpropanol are a little less effective.

Action on the intestine *in situ* has been determined for several of the esters previously described. These experiments were carried out on rabbits and dogs and the drugs were administered intravenously, intramuscularly or directly into the small intestine. The results obtained are in essential agreement with those obtained on the isolated intestinal segment. The compounds found most active on the isolated intestinal segment are also most active on the intestine *in situ*. After intravenous injection of the weak antispasmodic compounds, an occasional animal responded only by increased intestinal motility, as if these substances may exert a mild acetylcholine-like action.

2. *Action on Other Parasympathetically Innervated Structures.* a. Salivation. Effect on salivary secretion was determined in unanesthetized rabbits according to the method of v. Issekutz, modified as described below. The spasmolytic drug was injected intravenously 15 minutes after the subcutaneous injection of 10 to 20 mgm. of pilocarpine nitrate, at which time secretion was well established. If the secretion during this 15 minute period differed significantly in amount from that previously recorded for this animal during earlier control experiments, the experiment was discontinued. In computing the effect of the drug, the amount of saliva secreted before injection of the anticholinergic drug was subtracted from

TABLE 3

Effect of spasmolytic drugs on salivary secretion

COMP. NO.	DOSE	EFFECT ON SALIVARY SECRETION PER CENT CHANGE*
	mgm./kgm.	
6	1.000	+71
7	1.000	+14
8	1.000	−12
30	1.000	+10
31	1.000	+5
10	1.000	−60
	0.500	−40
11	0.500	+19
	0.100	−18
34	0.500	−11
	0.100	−10
35	0.500	−94
	0.100	−67
Atropine Sulfate	0.100	−96
	0.010	−77
	0.001	−70

* Average values, obtained from 6–10 experiments.
(+) Indicates increased secretion.
(−) Indicates decreased secretion.

the total secreted. Comparison was made with control experiments in the same animal, wherein the saliva secreted during the first 15 minutes was disregarded. Representative data are shown in table 3. The most active spasmolytic agents were also most effective in diminishing salivary secretion. However, the behavior of compound No. 11 was interesting in that the larger doses stimulated whereas small doses caused, on the average, a reduction in salivary secretion. Weak anticholinergic substances given in rather large doses (1.0 to 2.0 mgm. per kgm.) also increased salivary flow. This effect is not limited to esters containing thiophene inasmuch as compounds No. 6 and 7 also increased salivary flow. Salivation was sometimes noted in unanesthetized dogs following the administration of toxic doses of compound No. 11.

b. *Mydriasis.* Mydriatic action was determined in albino rabbits. All drugs were dissolved in distilled water and instilled directly into the conjunctival sac, the eye being held closed for one minute following instillation. Upon release of the eyelids, the excess drug was allowed to drain away. The size of the pupil was determined under direct illumination. The results obtained are shown in table 4. As noted above for salivary secretion, the most spasmolytic drugs were also most effective in producing mydriasis.

c. *Vascular Action.* The blocking action against the depressor effect of acetylcholine was determined in dogs by the method of Kühl. An amount of acetylcholine was injected intravenously to produce a 40–60 mm. Hg. fall in mean carotid blood pressure. The spasmolytic drug was then injected intravenously and the change in the response to the subsequent injections of acetylcholine recorded kymographically. The results obtained are shown in table 5. The most active spasmolytic drugs were most active in blocking the action of acetylcholine. Compounds Nos. 11 and 12 were comparable to atropine sulfate in their spasmolytic action and the most active one, Compound No. 12, approached atropine in its vascular blocking action. However, we believe the collected data indicates that no synthetic compound in this series is as effective as atropine in inducing anticholinergic side-reactions.

The pharmacological data outlined above indicates that the spasmolytic esters most effective in diminishing tonus of visceral muscle do cause reduction in activity of other organs innervated by the parasympathetic division of the autonomic nervous system. However, the intensity of this action is less than that of atropine. We have found intestinal tonus and the vasodepressor action of acetylcholine to be most easily diminished by the spasmolytic drugs in our series. Reduction of salivary secretion and increase in the size of the pupil occur with moderate doses and central nervous system disturbances only with the largest doses.

3. *Toxicity.* Acute toxicity was determined by intraperitoneal injection into albino mice. Results obtained are shown in table 6. An examination of the data indicates low toxicities for all acetates containing a single phenyl or cyclohexyl ring. The addition of a second phenyl ring results in a decided increase in toxicity. Of the compounds containing aryl or cycloalkyl substituents, the diphenylglycolates and phenyl-α-thienylglycolates are the most toxic and phenyl-

α-thienylacetates the least toxic. The most active compounds, the cyclohexyl-α-thienylglycolates are distinctly less toxic than phenyl-α-thienylglycolates and somewhat more toxic than phenyl-α-thienylacetates. Toxic symptoms produced are predominately those resulting from central nervous system stimulation. With lethal doses, death appeared to result from respiratory paralysis.

TABLE 4

Mydriatic action of spasmolytic drugs

COMP. NO.	CONCENTRATION	MYDRIASIS	DURATION OF RECORDED MYDRIASIS
	per cent		*hours*
6	1.000	++	1
7	1.000	+	1
8	1.000	+	1
30	1.000	+,++	½
31	1.000	+++	1
10	0.010	++	½
	0.050	++++	¾–1
11	0.010	++++	½
	0.050	++++	¾–1
34	0.010	++	½
	0.050	++++	½
35	0.010	+++,++++	1
	0.025	++++	1½
12	0.005	+++	2–3
	0.010	++++	2–3
Homatropine Hydrobromide	0.050	+++,++++	½–3
	0.100	++++	1½–3
Atropine Sulfate	0.005	++++	2–3
	0.001	+++	2–3

Maximum mydriasis is indicated by ++++.

Two glycolates, Nos. 11 and 12, were tested for toxic reactions in unanesthetized dogs following intramuscular or oral administration. The symptoms produced were restlessness, whining and barking, hyperpnea, muscle twitching, ataxia and vomiting. In severe intoxications, the above symptoms were followed by convulsions with marked opisthotonus at intervals during the seizure. In non-fatal intoxication this acute phase was followed by a period of light somno-

TABLE 5

Vascular anticholinergic action

COMP. NO.	DOSE	PER CENT EFFECT	DURATION
	mgm./kgm.		*minutes*
7	1.000	0–18	0–5
8	1.000	0–10	0–9
30	1.000	0	
11	0.250	80	>45
	0.050	60	44
12	0.050	40–90	140
	0.025	60	36
34	0.100	68	31
16	1.000	0	
17	1.000	0	
Atropine Sulfate	0.020	83–100	18–30
	0.010	50	35

TABLE 6

Acute toxicity of spasmolytic drugs intraperitoneal in albino mice

COMP. NO.	APPROX. LD_{50}	DOSE IN MGM./KGM.											
3	430	400	1/2*	440	6/10								
5	380	300	0/2	320	1/2	340	3/12	360	3/12	380	7/20		
6	490	400	0/2	440	0/4	480	1/4	500	4/4				
7	190	180	2/12	200	8/12	220	2/2						
8	320	260	0/10	280	10/30	300	11/30	320	16/30	340	19/30	360	9/10
9	500	400	0/2	440	0/2	480	1/3	500	1/2	520	2/2		
28	<60	40	1/2	60	1/2	100	2/2	140	2/2				
29	130	100	1/10	120	11/30	140	9/12	180	2/2				
30	440	360	2/10	380	4/10	400	4/14	420	8/18	440	1/4	460	4/4
31	200	120	0/4	140	1/4	180	3/4	220	1/2	260	3/3	300	3/3
10	110	80	3/17	100	10/32	120	15/24						
11	135	100	0/2	120	2/12	130	14/32	135	15/30	140	22/32		
32	140	100	0/6	120	1/4	140	6/12	160	2/2				
33	55	40	2/12	50	12/20	60	17/20	80	2/2	100	2/2		
34	135	120	3/10	130	4/10	135	9/20	140	15/20				
35	<60	40	2/2	60	4/6	80	14/16	100	12/14	135	10/10		
12	225	160	0/2	200	3/12	220	11/30	240	15/20				
37	220	160	0/2	180	1/2	200	0/4	220	7/12				
38	190	160	0/2	180	0/4	200	20/24	220	3/4				

* Dose in mgm./kgm. followed by number dead/number injected.

lence lasting for one to several hours. No evidence of physiological disturbances was noted during subsequent days. These findings may be taken as representative of the series and closely resemble those reported by Necheles et al. (2) for toxic doses of β-diethylaminoethyl diphenylacetate, and by Toda (11) for β-piperidinoethyl acetyltropate (Navigan).

Acute toxicity data obtained by intraperitoneal injection into albino mice suggest that β-dimethylaminoethanol gives acetic acid esters of the lowest toxicity, γ-diethylaminopropanol and γ-dimethylamino-β,β-dimethylpropanol of the highest toxicity. In general, similar results were obtained with the glycolates of these alcohols. It should be noted that for any one aminoalcohol, the phenyl-α-thienylglycolates were distinctly more toxic than the phenyl-α-thienylacetates. A similar relationship was found between β-diethylaminoethyl diphenylacetate and diphenylglycolate, the latter being distinctly more toxic. Meier and Hoffman (3) have shown the quaternary methochlorides or methobromides of a series of Trasentine-like esters to be more toxic than esters of the corresponding tertiary aminoalcohol. Compound no. 28, the only quaternary salt described in this series of compounds, was found to be much more toxic than the structurally similar tertiary amines, Nos. 8 and 30. The evidence indicates that for the anticholinergic esters described here toxicity is least with the β-dimethyl- or β-diethylaminoethyl di-α-thienylacetates or phenyl-α-thienylacetates.

SUMMARY

1. Anticholinergic spasmolytic compounds may be obtained by replacing the hydrogen atoms of various aminoalkyl acetates by phenyl, cyclohexyl, α-thienyl, or hydroxyl groups.

2. Highly active spasmolytic compounds are obtained with trisubstituted acetates wherein an hydroxyl group is combined with α-thienyl, phenyl or cyclohexyl groups.

3. The unsubstituted acetates of these aminoalcohols are cholinergic (muscarinic).

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ANESTHESIA XIX

The Anesthetic Action of n-Propyl Methyl Ether[1]

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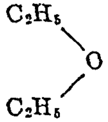
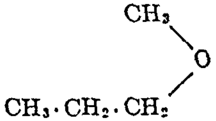
The two ethers which are generally employed as inhalation anesthetics are symmetrical ethers, namely diethyl and divinyl ethers. Our previous studies with mixed ethers such as cyclopropyl methyl (1), cyclopropyl vinyl (2), and isopropenyl vinyl (3) ethers, prompted us to study the simple isomer of ethyl ether, i.e., n-propyl methyl ether. The relationship of this compound to ethyl ether is apparent from the following formulas:



Ethyl Ether



n-Propyl Methyl Ether

Although n-propyl ethyl ether has been used as an anesthetic by Brown and Lucas (4), our survey of the literature revealed no comprehensive studies with n-propyl methyl ether as an anesthetic agent. Marsh working in Leake's laboratory studied the compound among other agents in correlating density and anesthetic activity. (5).

n-Propyl methyl ether is a volatile, colorless liquid with a characteristic ethereal odor: the boiling point is 39°C. and the specific gravity 0.726 at 16°C.

Anesthesia in the monkey. Three large *Macacus rhesus* monkeys were anesthetized with n-propyl methyl ether. The technic is described in detail in our former studies (1). The induction period was somewhat shorter than that with ethyl ether. Surgical anesthesia was uneventful; breathing was stertorous, deep and regular. Recovery from anesthesia was prompt; more rapid than with ethyl ether, but not so rapid as with propethylene (3). There was little excitation during the recovery period. The quantities of the agent employed were slightly less than those used to produce similar anesthetic syndromes with ethyl ether.

Anesthetic index (dog). The dogs employed were fed a diet of "Purina Chow" one week prior to the experiment and fasted twelve hours immediately before anesthetizing. At least two day intervals elapsed between anesthesias in the same animal. The procedure was identical with that employed in our cyprome ether (1) studies. The number of cubic centimeters of the agent per kilogram required to produce surgical anesthesia was divided into the volume required to produce respiratory arrest. The quotient was designated as the anesthetic index. The results are summarized in table 1.

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[1] The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Company of Cleveland, Ohio.

In our former studies with cyprome (1) and cypreth ethers (6) for comparative purposes, experiments were conducted with ethyl ether. In addition, six anesthetic index experiments were conducted with ethyl ether in these studies. The amount required to induce anesthesia with ethyl ether is 1.1 cc./kg. In summary, therefore, n-propyl methyl ether appears to be approximately 25 per cent more potent than ethyl ether upon induction. The toxicities and anesthetic indices of the two compounds are essentially the same.

BLOOD PRESSURE STUDIES (DOG). The effect of n-propyl methyl ether on the blood pressure was determined by anesthetizing the animal with ethyl ether.

TABLE 1

DOG NUMBER	SEX	WEIGHT	INDUCTION	RESPIRATORY FAILURE	ANESTHETIC INDEX
			cc./kg.	*cc./kg.*	
1	M	9.2	0.87	2.4	2.76
2	F	6.5	0.85	2.4	2.82
3	F	6.1	0.98	2.5	2.55
4	M	5.8	0.95	2.0	2.11
5	F	7.3	0.75	2.1	2.80
6	M	6.6	0.83	1.6	1.93
7	M	5.6	0.72	1.7	2.36
8	M	5.7	0.97	2.4	2.47
9	M	5.9	0.76	2.0	2.63
10	F	6.6	0.76	1.9	2.49
Mean			0.84	2.1	2.49

The blood pressure was determined in the usual manner by cannulating the carotid artery. The ether was removed and n-propyl methyl ether used as a substitute anesthetic agent. The respiratory tracings were made by means of a tracheal cannula and rubber tambour. The anesthesia was deepened to the point of respiratory collapse. The animal was then allowed to recover. The experiment was carried out on four animals and a typical tracing is shown in Chart 1.

ELECTROCARDIOGRAPHIC STUDIES (DOG AND MONKEY). Two monkeys and four dogs were cardioscoped under n-propyl methyl ether at the various planes of surgical anesthesia. No significant abnormalities were observed. After surgical anesthesia of 20 minutes duration, permanent tracings of the E.C.G. were made with the four dogs. A typical tracing, Lead II, is shown in Chart 2 of the dog before and under surgical anesthesia. The rates of heart beat were slightly increased; the R-spike slightly diminished in amplitude; the T-wave was neither inverted nor flattened.

EFFECT ON THE PERFUSED HEART (FROG). n-Propyl methyl ether was dissolved in Howell-Ringer's solution and perfused through the frog's heart *in situ*. Solutions containing 0.014 molar concentration produced a transient effect, lower concentrations produced no effect even upon prolonged perfusion. A typical tracing from one of six animals is shown in Chart 3.

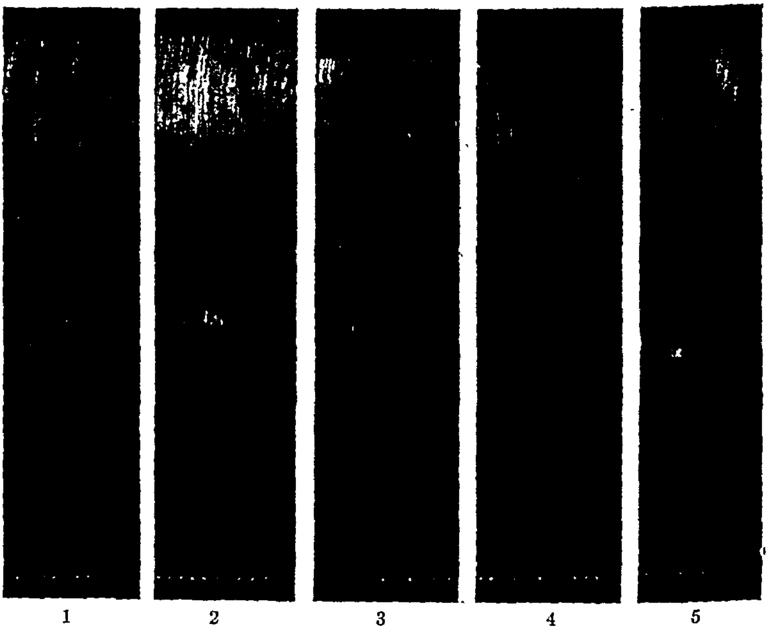
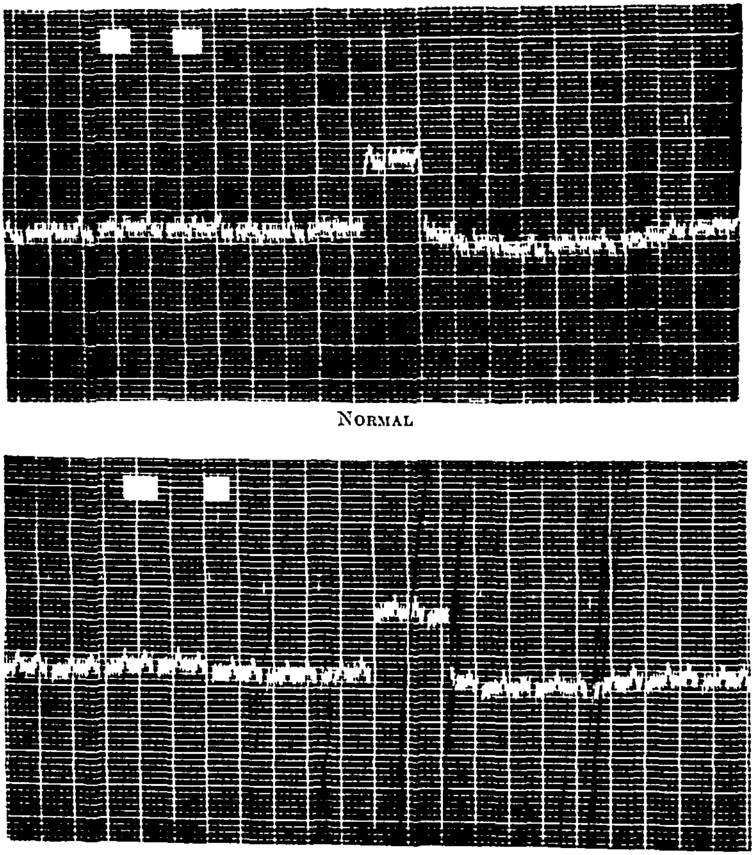


CHART 1. BLOOD PRESSURE OF DOG UNDER n-PROPYL METHYL ETHER ANESTHESIA
The upper tracing is respiration.
(1) Ethyl ether anesthesia.
(2) n-Propyl methyl ether anesthesia early stage.
(3) After 20 minutes deep surgical anesthesia with n-propyl methyl ether.
(4) Threatened respiratory arrest under n-propyl methyl ether anesthesia.
(5) Three minutes after removal of anesthetic cone.

LIVER FUNCTION TESTS (MONKEY). Six dogs were subjected to the bromsulfalein liver function test as set forth in our studies with cyprethylene ether (2). The dye excretion period was 30 minutes. Twenty-four hours after 60 minute anesthesias with n-propyl methyl ether, the dye excreted was not significantly different from the preanaesthetic rate.

BLOOD CHEMISTRY STUDIES (DOG). Three dogs were anesthetized to the surgical plane and then given more of the anesthetic until respiratory arrest occurred according to the anesthetic index technic. Prior to anesthesia and 24 hours later, blood samples were drawn for analysis. No significant changes in carbon dioxide-combining power or urea nitrogen were observed.

DELAYED ANESTHETIC DEATHS. Ten adult rats were anesthetized with n-propyl methyl ether to the surgical plane and maintained in this state for 30 minutes. Four animals were sacrificed at the end of 2 weeks and no significant findings were observed in the liver or kidneys. At the end of 3 weeks none of the animals had died or appeared to be in an unhealthy condition.


NORMAL

ANESTHESIA

CHART 2. ELECTROCARDIOGRAMS, NORMAL AND UNDER n-PROPYL METHYL ETHER ANESTHESIA TWENTY MINUTES (DOG LEAD II)

HISTOLOGICAL STUDIES OF VISCERA (RAT, DOG AND MONKEY). Four of the rats used in the delayed anesthetic death studies were sacrificed and their liver and kidneys were found to be free from significant changes. Three dogs were anesthetized for 90 minutes each on 3 alternate days. Two of the dogs were anesthetized by the open drop method and one by the closed circuit method with oxygen. On the sixth day after the first anesthesia liver biopsies were performed. There were no significant histological changes observed.

Two *Macacus rhesus* monkeys were subjected to the procedure of repeated anesthesias (60 minutes) as applied to the dogs. There were no significant histogical changes observed.

CLOTTING TIME AND HEMOLYSIS. The clotting time of the blood was determined in three normal dogs by the capillary tube method. The clotting time was between 30 and 45 seconds. Within the error of the experiment this period was neither diminished nor increased under surgical anesthesia with n-propyl methyl ether.

Volumes of 10 cc. of n-propyl methyl ether in varying concentrations in normal salt solution were maintained at 30°C. At 30°C. no hemolysis occurred over a 16 hour observation period in concentrations of 50, 100, and 150 mg. per cent. A saturated solution produced hemolysis within 1 minute similar to ethyl ether.

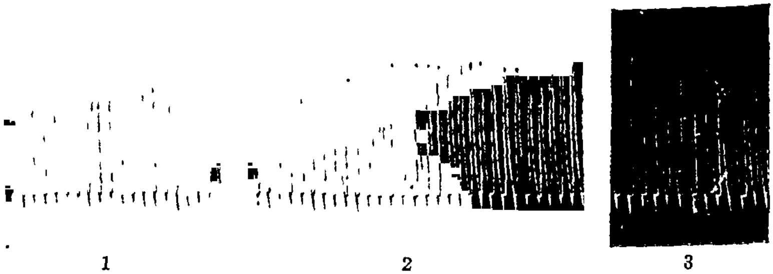


CHART 3. EFFECT OF n-PROPYL METHYL ETHER ON THE FROG'S HEART
(1) Howell-Ringer's Solution.
(2) Incipient Effect n-Propyl Methyl Ether.
(3) Same as "2" after 3 minutes.

PREANESTHETIC MEDICATION (DOG AND MONKEY). In monkeys, inducing n-propyl methyl ether anesthesia with nitrous oxide or cyclopropane-oxygen mixtures was uneventful. Preanesthetic medication with pentobarbital sodium or morphine-atropine was found to be compatible with n-propyl methyl ether anesthesia. Eight experiments in all were conducted on three animals.

PHYSICAL PROPERTIES. *Solubility in water.* A 10 cc. volume of n-propyl methyl ether was agitated vigorously with 100 cc. of water for two hours at 25°C. in a "Cassia Flask." The two liquids were allowed to separate for 12 hours and the volume of supernatant ether measured. The solubility was found to be 5.0 cc. per 100 cc. of water. Our value for anesthetic ether U.S.P. is 8.6 cc. per 100 cc. (1).

Oil/water coefficient. The oil/water coefficient was calculated from the data set forth by Carr et al. (7) on the relationship between water insolubility and oil/water coefficient. The value for n-propyl methyl ether is 10 ± 1, approximately double the value assigned to ethyl ether (1).

Inflammability range. n-Propyl methyl ether is isomeric with ethyl ether and will therefore have approximately the same inflammability range. This is about 2 per cent (lower limit) in air or oxygen (8).

Vapor pressure. The vapor pressure of n-propyl methyl ether determined at 28°C. in a nitrometer is 520 mm., that of ethyl ether at the same temperature is 597 mm. (9).

SUMMARY AND CONCLUSIONS

1. n-Propyl methyl ether, an isomer of ethyl ether is a volatile liquid, exhibiting anesthetic properties when administrated by inhalation to various species of animals.
2. The potency of n-propyl methyl ether is approximately 25 per cent greater than that of ethyl ether.
3. In the dog, n-propyl methyl ether anesthesia produces no functional liver damage as shown by the bromsulfalein test. In these experiments in the rat, dog and monkey anesthesias with n-propyl methyl ether produced no histopathological changes in the liver and kidneys.
4. Neither the monkey's nor the dog's heart showed any significant electrocardiographic changes under anesthesia with n-propyl methyl ether.
5. The blood pressure of the dog remains essentially unaltered under anesthesia with n-propyl methyl ether.
6. This isomer of ethyl ether compares very favorably with ether as an inhalation anesthetic in several species of animals. This first approximation of the anesthetic properties of n-propyl methyl ether, in our opinion, warrants its careful and judicious trial in man by skillled anesthetists. Extensive and intensive studies alone in human anesthesia will reveal whether or not this mixed ether will warrant a place in the armamentarium of the anesthetist.

Addendum. These experiments having been completed, we deemed that the properties of n-propyl methyl ether warranted its trial as an anesthetic in man. On September 22, 1945 at 10:30 a.m., one of us, (J. C. K., Jr.), administered n-propyl methyl ether to an anesthetist, Constance Black, by the open drop method. The induction period was about 8 minutes. Light anesthesia was continued for 3 minutes. The recovery was rapid and uneventful. The induction period was not marked by any excitation. During the hiatus in consciousness there were slight movements of the legs. There were no significant changes in blood pressure or pulse rate during the entire procedure. The subject stated that the vapors did not irritate the upper respiratory tract. Thirty cubic centimeters of the agent were used.

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METABOLIC STUDIES ON DERIVATIVES OF β-PHENYLETHYLAMINE

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The inactivation of sympathomimetic amines, particularly the β-phenylethylamine derivatives[1], by an amine oxidase enzyme system has received considerable attention since the report by Hare (1) in 1928 on the oxidative deamination of β-(p-hydroxyphenyl) ethylamine (*tyramine*) by an extract of rabbit liver. The properties of the system have been investigated by a number of workers, particularly Beyer (2), who has recently reviewed his studies on some fifty chemically related amines. His results are of value in interpreting the relation of structure to metabolism and to pharmacological activity.

The excretion of compounds of this type in the urine has also been the subject of much study since 1910 when Ewins and Laidlaw (3) demonstrated the conversion of β-(p-hydroxyphenyl) ethylamine to p-hydroxyphenyl acetic acid in the dog. The fate of α-methyl-β-phenylethylamine (*amphetamine, Benzedrine*) in man, dog, and rabbit has been studied rather extensively (4, 5, 6, 7). Slotta and Müller (8) have investigated the fate of β-(3,4,5-trimethoxyphenyl) ethylamine (*mescaline*) in rabbits and man, and Richter (4) and Beyer and Lee (9) have reported on the excretion of certain other of these compounds following their administration to humans.

The purposes of the present study were to determine the effect of amine oxidase on several unreported compounds of the β-phenylethylamine type, and to investigate the excretion in a small laboratory animal of a series of compounds having interesting structural relationships.

EXPERIMENTAL. *Amine oxidase studies.* Enzyme suspensions were prepared by homogenizing rabbit or guinea pig liver with three volumes of phosphate buffer (pH 7.2 or 7.7) in a Waring blendor. The homogenates were used either immediately or after dialyzing against distilled water at 0°C. for twenty-four hours.

Measurements were carried out in the conventional Warburg apparatus. In the side arm was placed 0.4 cc. of phosphate buffer containing 0.0025 mmol. of the amine hydrochloride. The liberated carbon dioxide was absorbed by 0.4 cc. of 20% potassium hydroxide on a small roll of filter paper. The main cell contained 1 cc. of the enzyme preparation. With the fresh liver preparations, 0.1 cc. of M/15 sodium cyanide was added to the suspension. This was replaced by 0.1 cc. of phosphate buffer when the dialyzed homogenates were used. The effect of sodium cyanide was similar to that of dialysis, in that both procedures led to greatly decreased consumption of oxygen by control samples of the enzyme preparation.

Excretion studies. In this work, 0.05 mmol. of the amine hydrochloride was injected subcutaneously into male white rats weighing from 250 to 400 grams. Five times this dose was given in two instances in order to determine whether the quantity of amine administered appreciably influenced the extent of excretion. Preliminary studies demonstrated that only insignificant quantities of amine were excreted after the first twenty-four hours; consequently the duration of the experiments was usually one day. To eliminate possible effects of diet, the animals were kept in metabolism cages without food but were given water *ad libitum* during the experimental period. An exception to this practice was made in some tests with N-ethyl-N-methyl-α-methyl-β-hydroxy-β-phenylethylamine (*Nethamine*), in which the influence of dietary variations was studied.

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[1] In the interest of consistency, and in order to bring out the structural interrelationships, the compounds will be named in this paper as derivatives of β-phenylethylamine. Common or commercial names will also be given where applicable.

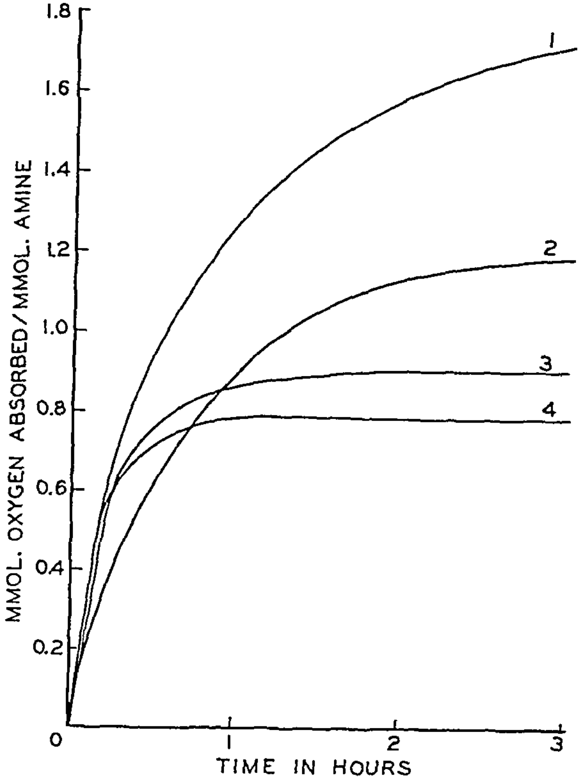


FIG. 1. ABSORPTION OF OXYGEN BY β-PHENYLETHYLAMINE AND DERIVATIVES IN THE PRESENCE OF LIVER HOMOGENATES

Curve 1: N-Methyl-β-methyl-β-phenylethylamine (*Vonedrine*).
Curve 2: β-Methyl-β-phenylethylamine.
Curve 3: β-Phenylethylamine.
Curve 4: β-(p-Hydroxyphenyl) ethylamine (Tyramine).

The picric acid colorimetric method of Richter (4) was used in carrying out the analyses. With all compounds, at least one series of tests was made in which an aliquot of the urine was boiled with hydrochloric acid before extraction in order to determine whether any of the compounds were excreted partially in a bound form.

DISCUSSION OF RESULTS. *Action of amine oxidase.* Four compounds, of the ten studied, were oxidized in the presence of the liver preparations; results with these four are presented graphically in figure 1. The other six compounds, α-methyl-β-phenylethylamine (*amphetamine, Benzedrine*), α-methyl-β-hydroxy-β-phenylethylamine (*Propadrine*), N-methyl-α-methyl-β-hydroxy-β-phenylethylamine (*ephedrine*), N-ethyl-N-methyl-α-methyl-β-hydroxy-β-phenylethylamine (*Nethamine*), N-ethyl-N-methyl-β-methyl-β-phenylethylamine, and β-hydroxy-β-methyl-β-phenylethylamine, were not attacked under the experimental conditions used.

The data confirm the principal conclusions of Beyer (2) concerning the influence of structure on oxidation. However, significant differences were observed in the relative rates and extents of oxidation of the four compounds attacked. It would appear that, under the present experimental conditions, the presence of the β-methyl group markedly affects the total oxygen uptake.

Excretion studies. Percentage values for urinary excretion of the compounds are given in table 1.

It will be seen that the four compounds having α-methyl substituents and the single di-β-substituted compound are excreted to a considerably greater extent than are those having neither of these structural characteristics. The excretion of the one tertiary amine that is unsubstituted in the α-position was found to be slightly higher than that of analogous primary and secondary amines.

It is interesting that two of the compounds, N-methyl-α-methyl-β-hydroxy-β-phenylethylamine (*ephedrine*) and β-hydroxy-β-methyl-β-phenylethylamine, appear to be excreted in a bound form, since the values were increased by boiling the urine with hydrochloric acid. There was no evidence of conjugation with any of the other compounds.

An increase in dose from 0.05 mmol. to 0.25 mmol. had no effect on percentage excretion in studies with two compounds, N-methyl-β-methyl-β-phenylethylamine (*Vonedrine*) and N-ethyl-N-methyl-α-methyl-β-hydroxy-β-phenylethylamine (*Nethamine*).

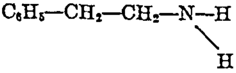
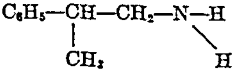
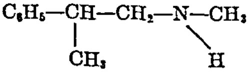
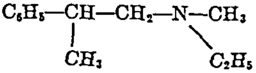
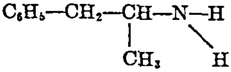
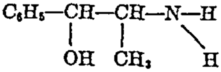
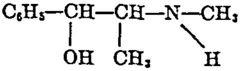
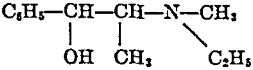
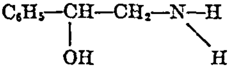
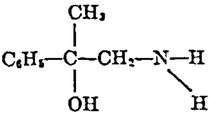
Effect of diet on excretion of Nethamine. In the case of N-ethyl-N-methyl-α-methyl-β-hydroxy-β-phenylethylamine (*Nethamine*), it was found that the addition of 1 or 3 per cent of ammonium chloride to the stock diet increased the excretion of the compound from a normal average of 20 per cent to an average of 30 and 33 per cent, respectively. Withholding of food, which might be expected to produce a mild acidosis, had a similar effect, leading to an average excretion of 25 per cent. Excretion of the amine was not affected significantly by a diet containing 1 per cent of sodium bicarbonate, but, when the bicarbonate was fed at a 3 per cent level, urinary excretion dropped to an average of 14 per cent of the dose.

RELATIONSHIP TO AMINO ACID METABOLISM. The influence of certain variations in chemical structure on the deamination of β-phenylethylamine derivatives and of α-amino acids seems worthy of mention. The pairs of compounds listed in table 2 bring out the principal structural relationships involved. In each case the carboxyl group of the amino acid replaces an α-hydrogen of the amine, and each of the amines contains a phenyl substituent in the β-position; otherwise the structures around the α- and β-carbon atoms are analogous.

It is seen that those structural modifications which do not interfere with the deamination of the amines are also without influence on the deamination or

TABLE 1

Excretion of β-phenylethylamine derivatives by the rat

COMPOUND	COMMON OR COMMERCIAL NAME	NO. OF ANIMALS	PER CENT OF DOSE EXCRETED IN URINE	
			Average	Range
 $C_6H_5-CH_2-CH_2-\underset{\diagdown H}{N}-H$	Phenylethylamine	6	1.7 ± 0.4	0.9–3.8
 $C_6H_5-\underset{CH_3}{CH}-CH_2-\underset{\diagdown H}{N}-H$		6	4.2 ± 0.5	2.3–5.8
 $C_6H_5-\underset{CH_3}{CH}-CH_2-\underset{\diagdown H}{N}-CH_3$	Vonedrine	6	3.0 ± 0.4	0.8–4.1
 $C_6H_5-\underset{CH_3}{CH}-CH_2-\underset{\diagdown C_2H_5}{N}-CH_3$		6	6.8 ± 1.0	3.1–9.9
 $C_6H_5-CH_2-\underset{CH_3}{CH}-\underset{\diagdown H}{N}-H$	Amphetamine, Benzedrine	6	14.8 ± 2.0	9.3–22.6
 $C_6H_5-\underset{OH}{CH}-\underset{CH_3}{CH}-\underset{\diagdown H}{N}-H$	Propadrine	6	36.0 ± 6.6	18.0–62.8
 $C_6H_5-\underset{OH}{CH}-\underset{CH_3}{CH}-\underset{\diagdown H}{N}-CH_3$	Ephedrine	4 7	Free: 27.2 ± 1.6 Total: 42.7 ± 4.9	22.0–30.0 32.0–65.6
 $C_6H_5-\underset{OH}{CH}-\underset{CH_3}{CH}-\underset{\diagdown C_2H_5}{N}-CH_3$	Nethamine	9	25.4 ± 2.8	10.0–40.1
 $C_6H_5-\underset{OH}{CH}-CH_2-\underset{\diagdown H}{N}-H$		6	3.1 ± 0.7	1.5–6.9
 $C_6H_5-\overset{CH_3}{\underset{OH}{C}}-CH_2-\underset{\diagdown H}{N}-H$		5 8	Free: 43.7 ± 2.9 Total: 65.3 ± 5.7	35.8–51.3 49.2–94.1

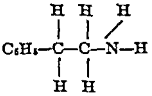
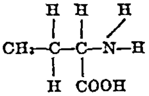
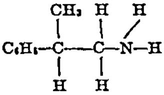
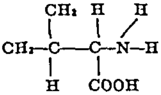
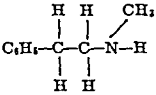
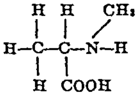
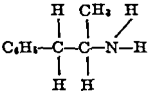
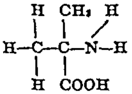
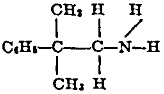
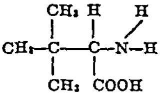
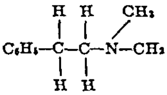
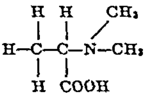
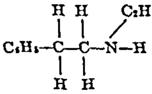
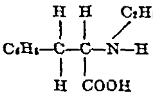
utilization of the corresponding amino acids. (As a partial exception, it has been shown that d(-) valine and d(-) isoleucine are not deaminated by the normal dog (10) and can not be used by the white rat for growth (11). However, the naturally occurring isomers are readily deaminated by the dog (10), and the d(-)

modifications are attacked by d-amino acid deaminase (12). It may be significant that the α-carbon atom in the amine of this type is not asymmetrically substituted.)

On the other hand, the structural variations which tend to prevent deamination of the amines have a similar effect on the corresponding types of amino acids.

TABLE 2

Relationship of structure to metabolism of amines and amino acids

	AMINE	METHOD & REFERENCE	AMINO ACID	METHOD & REFERENCE
Metabolized		Excretion (4) Enzymatic (19)		Enzymatic (12)
		Excretion (9) Enzymatic (2)		Enzymatic (12) Excretion (10)
		Enzymatic (2)		Enzymatic (15)
Not Metabolized		Excretion (4) Enzymatic (13)		Enzymatic (15) Excretion (16)
		Excretion (4)		Excretion (17)
		Enzymatic (2)		Enzymatic (15)
		Enzymatic (2)		Growth (18)

While Blaschko *et al.* (13) have shown clearly that amine oxidase is a separate entity from amino acid oxidase, it nevertheless appears that the two systems may have a common mechanism of action. Since the carboxyl group may be expected to have a rather profound influence on the enzyme-substrate relationship, there may exist a specificity in affinity without any fundamental differences in the mechanism of deamination (cf. Bergmann (14)).

SUMMARY

1. The principal conclusions of Beyer (2), concerning the deamination of β-phenylethylamine derivatives by amine oxidase, have been substantially confirmed.

2. Those compounds which are acted on by amine oxidase are also rather completely metabolized following administration to the rat.

3. Compounds containing a methyl group in the α-position are excreted to a considerable extent, from 10 to 65 per cent of the dose.

4. Two substituents in the β-position tend to decrease the extent of destruction in the body.

5. Tertiary amines may undergo destruction in the body if no other inhibiting groups are present.

6. Two compounds having a hydroxyl group in the β-position were found to be excreted by the rat partially in a bound form. The other compounds investigated appeared in the urine entirely in the free form.

7. The excretion of the N-ethyl-N-methyl-α-methyl-β-hydroxy-β-phenylethylamine (*Nethamine*) is increased by feeding a diet containing ammonium chloride and decreased by feeding a diet containing sodium bicarbonate.

8. Possible relationships to the deamination of amino acids are discussed.

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SOME TOXICOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF STREPTOMYCIN[1]

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Among the antibiotic agents with high chemotherapeutic activity streptomycin (1, 2, 3, 4, 5, 6, 7, 8) has recently attracted considerable attention. This is due not only to its effectiveness against gram-negative bacteria which are not influenced by penicillin, but also to the relatively low toxicity which distinguishes it favorably from streptothricin, an antibiotic with otherwise very similar chemotherapeutic properties, but with a systemic toxicity sufficiently pronounced (9, 10) to make its clinical parenteral use inadvisable.

With the exception of a short preliminary report (8), no systematic study of the toxicological and pharmacological properties of streptomycin has yet been published and the data presented here are intended to serve this purpose.

This study was begun in October, 1943 using a crude preparation of rather low potency, containing in 1 mgm. of solids the equivalent of only 30 micrograms of streptomycin base (30 units).[2] Subsequently, material of greater purity and higher potency became available and finally pure streptomycin was included in the investigation. However, in view of the relative scarcity of pure streptomycin which at the present time makes impractical its use on a large scale, most of our experiments were conducted with streptomycin concentrates identical with those used in man. A total of approximately one billion units (equivalent to 1 kgm. of pure streptomycin base) were required for this investigation, including approximately 50 grams of pure streptomycin; the potency of the streptomycin concentrates ranged from 30 to 600 micrograms of streptomycin base per mgm. solids.

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[1] The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Merck Institute for Therapeutic Research, Rahway, New Jersey.

All streptomycin used in this study was supplied by the Research Laboratories of Merck & Co., Inc.

[2] One unit of streptomycin was originally defined as that quantity which will inhibit a given strain of *E. coli* in 1 cc. of nutrient broth or agar. The potency of a streptomycin concentrate is now expressed in terms of its equivalent in weight of pure streptomycin base, 1 microgram of the latter being equal to 1 unit in the former nomenclature. In this paper all doses expressed in terms of weight refer to weight of streptomycin base administered and not to the actual weight of streptomycin concentrate. The following terms will be used: "pure streptomycin" for the chemically pure material with a potency of approximately 800 units per mgm.; "streptomycin concentrate" for preparations other than pure streptomycin, varying in potency from 30–600 units (30–600 micrograms streptomycin base) per mgm. solids. The name "streptomycin" will be used when the discussion deals with properties common to both pure streptomycin and streptomycin concentrates.

TOXICITY STUDIES. The acute and chronic toxicity of streptomycin was studied in 185 frogs, 16,000 mice, 520 rats, 295 guinea pigs, 5 dogs and 42 monkeys. In view of a considerable variation in the toxicity of individual lots, care was taken to use material from the same lot for each group of experiments, particularly when data for the comparison of the toxicity in different species were desired. Only in experiments which extended over many weeks or months was the use of different lots unavoidable, due to insufficient supplies.

The animals were kept in air-conditioned quarters at a relative humidity of 50% and a temperature of 75°F. The mice and rats were of uniform strains obtained from the Carworth Farms. The mice (CFW strain) weighed between 15 and 20 grams, the rats (Wistar strain) 120 to 150 grams; for a limited number of experiments weanling rats were used. Mice and rats were fed a nutritionally complete laboratory diet. The animals were given free access to food and water, except as otherwise stated. Guinea pigs with an average weight of 375 grams were obtained on the open market and kept on a mixed diet of grain and greens. The monkeys were Macacus rhesus weighing between 3 and 6 kgm. They were kept under observation for at least three weeks before being placed on test; animals which during this time had lost weight or otherwise did not appear healthy were rejected. Monkeys were fed a mixed diet, consisting of Purina pellets, fresh fruits and vegetables. The dogs were mongrels weighing 8 to 11 kgm. and were maintained on a mixture of Gaines dog meal, fresh horse meat and milk.

Acute toxicity. Mice: Typical mortality curves following subcutaneous and intravenous administration of pure streptomycin and streptomycin concentrates are shown in figure 1. Ten animals were used for each dose level of the streptomycin concentrates and five animals for each dose level of pure streptomycin. In this, as in all other experiments, subcutaneous injections were made under the abdominal skin, using a No. 25 needle and an average volume of 0.3 cc. for the injection. Intravenous injections were made at a uniform rate of 0.1 cc. per minute into the tail vein of mice or the femoral vein of rats. All animals were observed continuously during the first hour after injection, at hourly intervals for the next ten hours and daily thereafter up to ten days.

The data shown in figure 1 reveal a rather pronounced difference between the toxicity upon intravenous and subcutaneous injection, the former being five to ten times greater. Almost immediately after the completion of the intravenous injection of a toxic dose the animals exhibited respiratory difficulties and lost consciousness; they apparently died from asphyxia, since the heart continued to beat for several minutes after cessation of the respiration. Death almost invariably occurred within five minutes; no delayed deaths were observed in animals surviving this critical period.

Following a subcutaneous injection, the animals soon became restless, breathed with difficulty, lost their balance and lapsed into a coma in which they occasionally remained as long as one day prior to death. However, once the mice recovered from the initial effect of a near-fatal dose they remained alive and well. This is in striking contrast to the course of streptothricin poisoning, which is characterized by an apparent complete recovery from an initially non-fatal single dose but is often followed by a large number of late deaths due to progressive renal insufficiency.

In view of the marked difference between the subcutaneous and intravenous toxicity and the fact that streptomycin is very rapidly excreted (11) a comparison was made between the acute toxicity of a single dose and that of the same quantity distributed over several smaller doses. In one experiment, the lethal toxicity of a single subcutaneous injection was compared with that of eight divided doses of the same lot injected at three hour intervals. With the latter schedule, the mice tolerated an amount four times greater than the single L.D. 50. In another experiment the toxicity of a single subcutaneous dose was compared with that of the same amount given at six hour intervals over a period of eighteen hours. While the single subcutaneous administration of 750 mgm. per kgm. caused death of 9 out of 10 mice, 3 out of 10 tolerated as much as 1300 mgm. per kgm. of the same lot when injected in three equally divided doses.

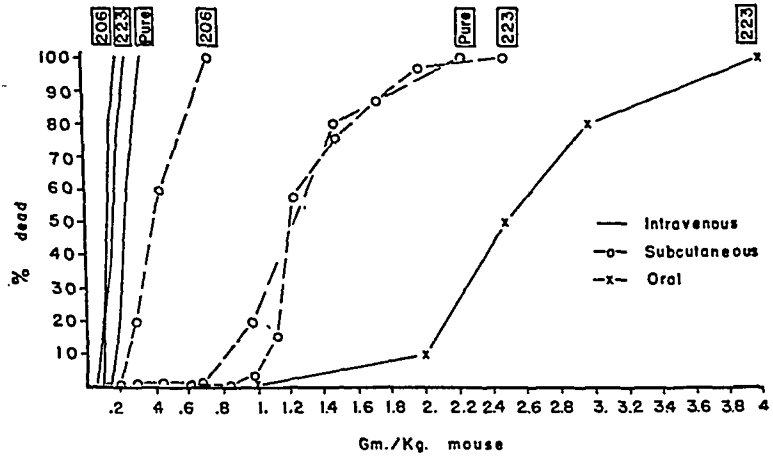

FIG. 1. TOXICITY OF STREPTOMYCIN IN MICE
The numbers in the rectangle indicate the lot of streptomycin concentrate used

As previously mentioned, the acute toxicity of streptomycin in mice shows wide variations which are apparently not related to the potency (purity) of the lot used. This is illustrated in figure 2, which shows the acute L.D. 50 of 200 different lots of streptomycin in relation to their potency. While the L.D. 50 of the majority of those lots ranges between 350–700 mgm. per kgm., it may be seen that some of the samples with a potency as high as 400 to 450 micrograms streptomycin base per mgm. have an L.D. 50 as low as 150–250 mgm. per kgm. while others with low potencies of 100 to 200 micrograms streptomycin base per mgm. are tolerated at levels of about 1000 mgm. per kgm. Differences in the rate of absorption may play a part in the lack of correlation between potency and toxicity, as can be seen from the following experiments.

Groups of mice were injected subcutaneously with 750-1500 mgm. per kgm. of a relatively pure material (about 800 micrograms streptomycin base per mgm.) and with the same number of units of a less potent preparation (300 micrograms streptomycin base per mgm.); the L.D. 50 of the latter was 1500 mgm. per kgm.

as compared to 750 mgm. per kgm. for the more highly purified material. Within two to three minutes the mice injected with lethal doses of the high potency material showed severe respiratory depression and died in less than ten minutes; those injected with similar doses of the less purified concentrates exhibited the first signs of toxicity after thirty minutes and died after two hours. This indicates that the rate of absorption plays a rôle in determining the subcutaneous toxicity, although it is not the only factor influencing it. This is demonstrated by the fact that a similar lack of correlation between potency and toxicity of different lots was observed after intravenous injection (fig. 3). This shows a comparison of the acute subcutaneous and intravenous toxicity of 28 lots of streptomycin varying in potency from 35 to 800 micrograms streptomycin base per mgm. Here also there is no consistent relationship between the potency of a lot and its intravenous L.D. 50, although the rate of absorption is eliminated as a factor. However, the difference in toxicity between samples of like potency are much less pronounced after intravenous administration.

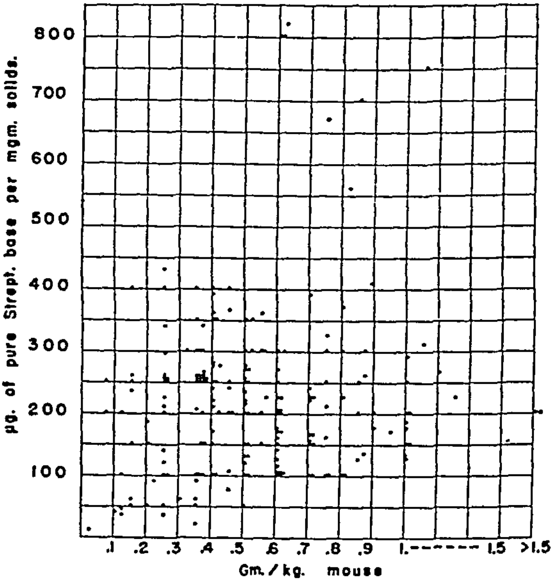

FIG. 2. ACUTE SUBCUTANEOUS TOXICITY IN MICE OF 200 LOTS OF STREPTOMYCIN
Abscissa: Subcutaneous L.D. 50 expressed in terms of grams streptomycin base.
Ordinate: Potency of preparation expressed in terms of micrograms of streptomycin base per mgm. solids.

The acute oral toxicity of streptomycin was determined in only a small number of experiments, since it soon became apparent that factors other than those responsible for the toxicity upon parenteral administration greatly influenced the results. Although mice failed to die after doses of 5000 mgm. per kgm. of pure streptomycin, the administration of 1000–3000 mgm. per kgm. of streptomycin concentrates was fatal. While in the late stages of poisoning the syptoms resembled those after parenteral administration (respiratory depression and coma) the earlier stages were characterized by pronounced restlessness and excessive thirst. Upon autopsy the gastro-intestinal tract was found to contain an unusually large amount of fluid and showed multiple hemorrhagic lesions. These may have been due to the local irritating properties of streptomycin as well as the high concentration of the solutions necessary to administer an orally lethal dose. In order to examine this possibility, groups of 10 mice were given by stomach tube doses of sodium chloride corresponding in concentration and volume to doses of a streptomycin concentrate which were lethal. The doses of NaCl given were 1.5, 3, 3.75, 4.5 and 6 grams per kgm., corresponding to

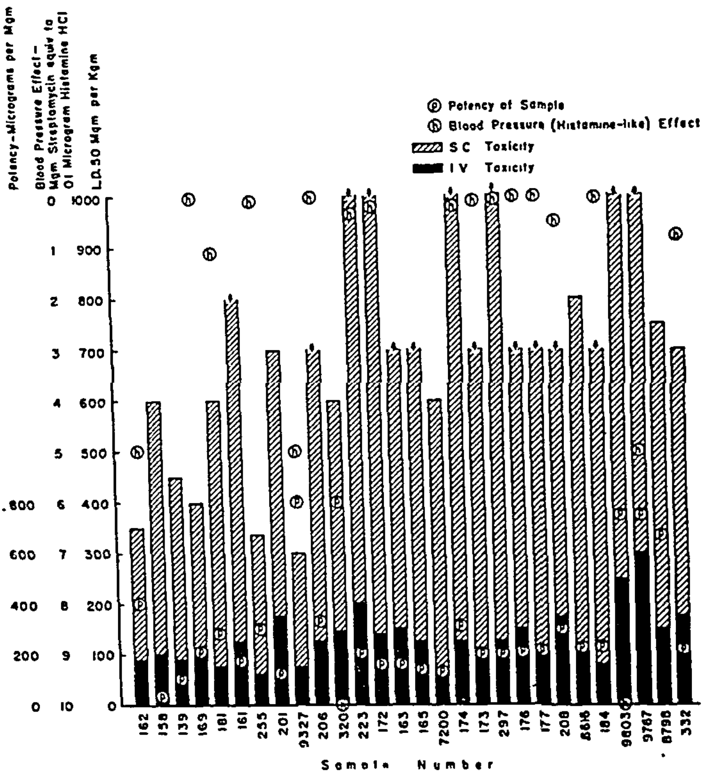

FIG. 3. POTENCY, BLOOD PRESSURE EFFECT AND TOXICITY OF 28 STREPTOMYCIN SAMPLES

Light-shaded column: subcutaneous toxicity.

Dark column: intravenous toxicity.

Circles with letter h indicate degree of depressor activity in equivalents of histamine hydrochloride.

Circles with letter p indicate potency in terms of micrograms streptomycin base per mgm. solids.

doses of 2.5, 5, 6.25, 7.5 and 10 grams of streptomycin concentrate per kgm., equivalent respectively to 1000, 2000, 2500, 3000 and 4000 mgm. streptomycin base. While with streptomycin concentrate the first deaths occurred at 5 grams per kgm. and the L.D. 100 was reached at 10 grams per kgm. deaths in animals dosed with comparable salt concentrations started at 6 grams per kgm. (corresponding to 10 grams per kgm. of streptomycin concentrate). It therefore appears that toxicity following oral administration of large amounts of streptomycin is influenced by the high osmotic concentration of these solutions. This view is further supported by the observation that the concentration of streptomycin in the blood of mice dying from excessive oral doses (3000–4000 mgm. per kgm.) is relatively low (60–120 micrograms streptomycin base per cc.). This is considerably less than the concentration found after intravenous injection of large but non-fatal doses.

Frogs: The acute toxicity of several streptomycin concentrates and pure streptomycin was determined in Rana pipiens by injection into the abdominal lymph sac of 0.2–0.8 cc. of aqueous solutions containing 25–100 mgm. of streptomycin base per cc. Five frogs were used at each dose level. After injection the frogs were placed in small individual metal containers with screen bottoms which were kept approximately 1 cm. below the water level of a thermostatically controlled tank filled with constantly changing water at 25°C. Continuous observations were made during the first hour after injection and at twelve hour intervals for the following three days.

Within one minute a reddish area appeared at the site of injection, increasing in diameter and duration with the dose. Within approximately ten minutes the frogs became partially paralyzed. Although still responding to tactile stimuli, they exhibited increasing difficulty in righting themselves when placed on their backs. Within fifteen to twenty minutes a complete motor paralysis had developed; no respiratory movements were evident. However, in contrast to warm blooded animals which in this stage of poisoning would have died from respiratory failure, frogs often completely recovered after two to three days when kept partially submerged in water.

The doses of streptomycin tolerated by frogs were approximately one and one-half times larger than the subcutaneous and fifteen times larger than the intravenous L.D. 50 for mice, but occasional lots of streptomycin concentrates were as toxic for frogs as for mice.

Guinea pigs: A streptomycin concentrate with an (s.c.) L.D. 50 for mice of 600 mgm. per kgm. had in guinea pigs an L.D. 0 of approximately 200 mgm. per kgm., and an L.D. 50 of 400 mgm. per kgm., and an L.D.100 of approximately 700 mgm. per kgm. (s.c.).

Monkeys: No acute experiments were carried out in monkeys, due to a limited supply of these animals as well as of the drug. However, in the course of a large number of chronic toxicity experiments it was observed that in this species intravenous and occasionally even subcutaneous injection of 30–70 mgm. per kgm. was followed by marked respiratory depression, which at times necessitated the use of artificial respiration. These doses are considerably below those effecting comparable toxic signs in the mouse.

Chronic toxicity. Mice: Administration of streptomycin to mice for a prolonged period of time was carried out on only a limited scale, because the relatively high incidence of deaths from non-specific causes seriously interferes with the interpretation of results.

A group of 40 mice was given subcutaneous injections of 150 mgm. per kgm. per day for six days in three equally divided doses; another group of 20 mice received 1000 mgm. per kgm. in five divided doses daily by subcutaneous injection for six days. All mice remained normal during the period of drug administration and a subsequent ten day observation period. Other groups of mice received for one month 150, 300, 600 and 1500 mgm. per kgm. per day, mixed with the diet. All animals remained normal. No gross pathological findings were observed in any of these experiments.

Rats: In rats, the following chronic toxicity studies were performed. A group of 30 rats with an average body weight of 215 grams received by subcutaneous injection 100 mgm. per kgm. streptomycin in three divided doses daily for seventy-two days. No toxic signs were observed except for an occasional local induration or sore at the site of injection. The average gain in body weight of the treated animals was 18%, compared with 19% for the controls and 28% for a group which had been dosed with streptomycin for only 16 days and had been kept under observation for the remainder of the time. All groups were sacrificed at the end of the seventy-two day period; no pathologic changes were found upon gross anatomical and histological examination.

Two other groups of 20 rats received 400 mgm. per kgm. daily for eight days by subcutaneous injection and 400 mgm. per kgm. daily for six days by intravenous injection. No adverse signs were observed in either group.

Two groups of 60 weanling rats were given streptomycin incorporated in the diet at dose levels of 300 and 900 mgm. per kgm. of body weight.[3] Both groups grew at a rate only slightly below that of the controls. About the sixth week the rats receiving 300 mgm. per kgm. per day exhibited nervous hyperexcitability. The group receiving 900 mgm. per kgm. per day developed these nervous signs within twenty-four hours after the beginning of the test, but remained otherwise normal. At autopsy no abnormalities were found.

Guinea pigs: A group of 65 guinea pigs with an average body weight of 375 grams was given for a period of six to eight weeks daily subcutaneous injections of 20, 30, 40, or 60 mgm. per kgm. of streptomycin in three divided doses. Fifteen animals were used on each dose level and one group of 5 animals served as controls. With two exceptions, the animals gained an average of 100 grams and appeared to be well throughout the test; 2 animals on the 40 mgm. per kgm. level died fifteen days after the beginning of the test, apparently from non-specific causes. Gross pathological examination revealed no effects attributable to the drug.

Monkeys: A group of 4 monkeys was given daily subcutaneous injections of 25 mgm. per kgm. of streptomycin for sixty-six consecutive days. The material used for this experiment had to be taken from seven different lots, ranging in potency from 50 to 170 micrograms streptomycin base per mgm. and having an L.D. 50 for mice of 250–800 mgm. per kgm. All monkeys remained well throughout the experiment, gained weight and showed no adverse effects, other than a slight anemia and skin irritation at the site of injection. In particular, there was *no significant pathologic effect upon the kidney*, as judged by frequent determinations of blood urea, chemical and microscopic examination of the urine, and histological examination of the tissue.

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[3] We are indebted to Dr. Gladys A. Emerson and Miss Dorothy G. Smith for permission to use these data.

Since in a comparison of the toxicological properties of streptothricin and streptomycin (determined earlier in this laboratory) it had been found that a five day period of drug administration was sufficient to produce in the monkey pathologic changes characteristic for the particular agent, it was decided to limit subsequent monkey experiments to five days of streptomycin administration followed by an observation period of at least ten days. Accordingly, a total of 15 monkeys was given intravenous injections of 25, 50, or 200 mgm. per kgm. daily for five days. The two lower dose levels were administered in three portions, while the 200 mgm. per kgm. dose was distributed over six injections daily in order to minimize the respiratory depression resulting from intravenous administration of this amount. The material used in these experiments were pure streptomycin (800 micrograms streptomycin base per mgm.) and a streptomycin concentrate (400 micrograms streptomycin base per mgm.). There was essentially no difference in the results obtained with the two preparations. *The animals injected with the two lower doses remained normal* through the five day experimental and ten day observation period; no evidence of renal damage was found, but there was an occasional transient impairment of hepatic function as judged by the bromsulphalein retention test. One monkey which received 200 mgm. per kgm. daily died on the second day from respiratory paralysis following the injection; the other survived the fifteen day period, but showed a transient proteinuria with a peak value of 0.9 gram protein per liter of urine.

A group of 16 monkeys received streptomycin concentrates by subcutaneous or intramuscular injection in doses of 10, 50, 100, or 200 mgm. per kgm. In animals on the two lower dose levels no deleterious clinical effects were observed during the five day experimental and ten day observation period, except for occasional tenderness and sores at the site of injection. With the two higher dose levels there were likewise no significant changes during the dosing period, but during the following ten day observation period 3 of the 12 animals showed a transient proteinuria with peak concentrations of 2.6 to 4.5 grams per liter; bromsulphalein retention was observed in 2 of the monkeys.

All monkeys were autopsied 12–20 days after the last injection and gross and microscopic examinations were performed. In several animals, especially those injected intramuscularly, areas of necrosis with evidence of repair were present at the sites of injecton. The most characteristic pathological finding attributable to the streptomycin treatment was the presence of a fatty metamorphosis in the liver and, less often, in the kidney. Only doses of 25 mgm. per kgm. or higher produced this effect. No decrease in the glycogen content of the liver was observed.

In order to determine whether the pathologic changes in the liver and kidney just described constituted a permanent damage or would disappear after the administration of streptomycin had been discontinued, 8 monkeys were dosed intravenously in the usual manner for five days with 25 mgm. per kgm. per day and were sacrificed in pairs at the end of the dosing period, after ten additional days, after one month and after two months of observation. Hepatic and renal function tests, hematologic examinations, blood urea determinations and chemical and microscopic analyses of the urine were performed.

There were only minor and transient functional changes and all animals remained outwardly healthy. Upon autopsy, the monkeys sacrificed one day after the last streptomycin injection showed a moderate amount of fat in the liver but none in the kidney. After ten days a larger amount was present in the liver and fat was also found to a slight degree in the kidney. After thirty days the fat had completely disappeared from the latter and was definitely less in the liver than previously. After sixty-six days all pathologic changes had disappeared. The conclusion seems therefore justified, that the fatty metamorphosis in the liver and kidney of monkeys resulting from a five day parenteral administration of streptomycin is reversible. Whether this will be the case when the drug is administered over a much longer time or at a higher dose remains to be determined. The fact, however, that the monkeys injected with the same dose for sixty-six consecutive days had shown no pathologic changes exceeding those found after a five day administration of the drug makes it unlikely that the fatty metamorphosis observed after a few days is progressive and results in a severe and irreparable damage.

Dogs: Five dogs were injected subcutaneously or intramuscularly for twenty days with 50 or 100 mgm. per kgm. daily in three divided doses. After one and one-half to two weeks all animals developed proteinuria of varying degree, reaching in 2 dogs 11.5 and 46 grams of protein per liter respectively; those animals which showed the most pronounced proteinuria also had a significant decrease in serum protein. The appearance of casts, epithelial cells and leucocytes also became a constant finding. At autopsy, the liver of one dog which had received the larger dose was distinctly yellow and the median lobe had pale areas suggestive of necrosis. Pale streaks were present in the cortex of the kidneys. Sudan IV stains revealed considerable lipoid in the central portion of the nephrons, but only a relatively small amount in the liver. A slight degree of tubular necrosis was observed in one dog, which had shown severe proteinuria.

Three of the 5 dogs also developed a change in gait and posture, which suggested a labyrinthine or cerebellar disturbance. The head was usually cocked to one side and they tended to walk with an unsteady, wavering gait. An impairment of their auditory acuity was suggested by failure to respond normally to sudden noises. Hinshaw and Feldman (12) have recently reported similar findings in several patients which received large doses of streptomycin for prolonged periods.

Influence of rate of injection: In view of the probability that streptomycin might be given clinically by slow intravenous drip, this method of administration was investigated in 2 monkeys weighing 3.9 kgm. and 3.8 kgm. respectively. The animals were lightly anesthetized with nembutal (30 mgm. per kgm.) and aqueous streptomycin solutions of varying concentrations were infused at constant speeds. The dosage of streptomycin was 200 mgm. per kgm. per hour and 120 mgm. per kgm. per hour respectively. The first monkey died after a total of 440 mgm.; the second monkey tolerated a total of 1920 mgm. over a forty-two hour period. No significant changes in the heart rate, respiratory rate and temperature were observed in this animal, whereas the animal receiving streptomycin at the higher rate of infusion (200 mgm. per kgm. per hour) exhibited respiratory depression of a type similar to that observed after intravenous injection of streptomycin into smaller animals.

Rats anesthetized with 30 mgm. per kgm. of nembutal were given streptomycin infusions into the femoral vein at the rates of 600, 180 and 40 mgm. per kgm. per hour. Respiratory movements and carotid blood pressure were recorded in all experiments. At the highest rate of infusion the respiration stopped within approximately six minutes, and at the intermediate rate within approximately twenty minutes. The heart continued to beat for four to six minutes after cessation of the respiration. The rats receiving the drug at the slowest rate showed no noticeable adverse effects when the experiment was terminated after one and one-half hours, after administration of approximately 60 mgm. of streptomycin per kgm. On the other hand doses of 40 or 50 mgm. per kgm. were fatal when given at more rapid rates of injection.

Local effects. The local effects of sixty-five different lots of streptomycin including the pure material were tested in rabbits, guinea pigs, monkeys and dogs by application to the eye and buccal membranes, by injection into the depilated abdominal skin and by intrapleural, subcutaneous and deep intramuscular injection. The pH of the solutions varied between 6 and 7.

Eye: The experiments on the eye were conducted by instilling a solution containing 8 mgm. of streptomycin base per cc. into the conjunctival sac of lightly anesthetized rabbits and permitting it to remain for periods up to thirty minutes. In another series of tests an ointment containing 1 mgm. of streptomycin in 1 gram of an inert opthalmic ointment base was applied to the eye with a glass rod. Continuous observations were made during the first hour following the application and at less frequent intervals during the next five days. Except for an occasional redness of the conjunctivae which appeared shortly after the application and persisted up to twelve hours, neither the solution nor the ointment produced any other noticeable effects. This is in striking contrast to the action of streptothricin, which in addition to a much more violent immediate reaction produces a delayed effect, consisting of a suppurative conjunctivitis with destruction of the cornea (9).

In spite of considerable variations in potency, the streptomycin lots used for these experiments differed only slightly in the degree to which they produced the transient irritation; it was also observed with the pure material and seemed to depend as much upon the sensitivity of the individual test animal as upon the properties of the particular lot.

Buccal membranes: The effects of topical application were determined by applying the above ointments to the buccal membrane of dogs for fifteen minutes. No immediate or late effects were observed nor did the animals exhibit any signs of discomfort.

Intrademal injection: The effects of intradermal administration were tested by injecting into the depilated abdominal skin of guinea pigs 0.1 cc. of buffered solutions containing 1, 2 or 4 mgm. of streptomycin per cc. Considerable variations were found among sixty-five lots examined in this manner. As a rule, however, the effects were not serious, consisting of a slight reddening at the site of injection, occasionally followed by formation of a reddish and slightly upraised small blister which disappeared within one to two days, leaving a dry, pinpoint sized crust in the center of the affected area. Formation of a heavier crust and a persistent thickening at the site of injection was noted only once with the 1 mgm. per cc. concentration and only five times with the 4 mgm. per cc. concentration and was independent of the potency of the material. It was also observed with pure streptomycin.

Intrapleural injection: In view of the possible use of streptomycin in the treatment of empyema, three groups of two rabbits each were given a single intrapleural injection of 1, 10 or 100 mgm. per kgm. of a lot which had shown only very slight local irritation when tested by instillation into the eye and by intradermal injection. The concentrations of the solutions were such that each animal received the selected dose in 1 cc. per kgm. body weight. All animals were sacrificed and examined on the fourth day after injection. In the rabbits treated with the lowest dose a slight amount of pleural fluid was found and the diaphragm was slightly congested. More fluid and greater congestion were induced by the larger doses and the animals injected with 100 mgm. per cc. per kgm. showed small areas of hemorrhage and fibrinous adhesions between the lung, diaphragm and pleural wall.

Subcutaneous injection: Following subcutaneous injection, local irritation consisting of edema, hemorrhage and necrosis with or without ulceration of the epidermis was occasionally observed. These reactions were found in every species examined and varied greatly in intensity with the different lots; they were definitely more severe with the earlier, less purified material, but were also present with high potency concentrates and pure streptomycin.

Intramuscular injection: Essentially similar reactions were produced by intramuscular injections. Neither the subcutaneous nor the intramuscular injection seemed to be accompanied by a marked degree of pain, although dogs injected with concentrated solutions of streptomycin (100 mgm. per cc.) into the muscles of the hind legs exhibited signs of local discomfort and limped for several hours after the injection.

Intravenous injection: No signs of local damage were found in the veins following repeated intravenous injections of streptomycin solutions in concentrations up to 68 mgm. per cc. Following a forty-two hour continuous infusion of a solution containing 3.2 mgm. of streptomycin per cc. one monkey showed evidence of thrombus formation at the site of the cannula due possibly to the mechanical trauma.

The various manifestations of local irritation were observed after single as well as repeated administration of the drug. They were generally more pronounced with material of low potency and higher ash content, although they were also noted with pure streptomycin. Samples containing less than 0.1 mgm. of streptomycin base per mgm. of solids almost invariably caused severe local reactions. It appears that in such samples the degree of local as well as systemic side-effects is directly related to the potency of the material.

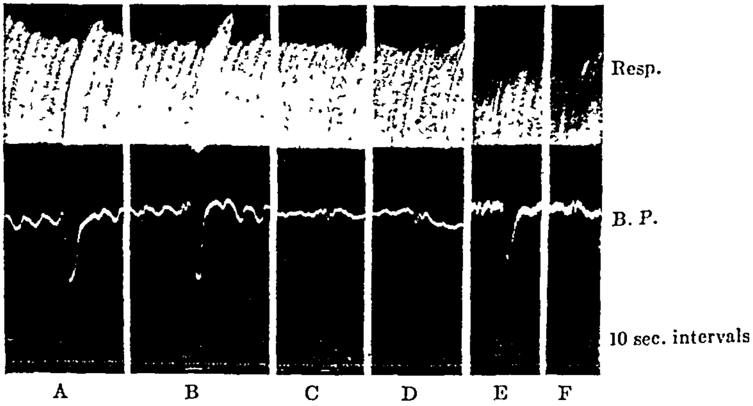

FIG. 4. COMPARISON OF THE CIRCULATORY EFFECT OF DIFFERENT LOTS OF STREPTOMYCIN IN A CAT

(30 mgm. per kgm. Nembutal i.p.)

A: 0.003 mgm per kgm. histamine hydrochloride.
B: 5000 units (5 mgm. streptomycin base) per kgm. of streptomycin concentrate Lot 169
C: 5000 units (5 mgm. streptomycin base) per kgm. of streptomycin concentrate Lot 162.
D: 10,000 units (10 mgm. streptomycin base) per kgm. of streptomycin concentrate Lot 162.
E: 10,000 units (10 mgm. streptomycin base) per kgm. of streptomycin concentrate Lot 8798.
F: 10,000 units (10 mgm. streptomycin base) per kgm. of pure streptomycin hydrochloride.

Note the similarity of A, B, and E and the absence of blood pressure effect with comparable doses in C, D, and F.

PHARMACODYNAMIC EFFECTS. The acute pharmacodynamic effects of streptomycin depend to a large degree upon the purity of the preparation. This is due to the fact that streptomycin concentrates often contain contaminants, which mask the properties of the pharmacodynamically far less active streptomycin. One such contaminant has histamine-like properties.

Circulatory effects. Streptomycin concentrates: Lots containing the histamine-like factor to any marked degree cause in a variety of animal species (mice, rats, rabbits, guinea pigs, cats, dogs) almost all the effects of histamine, particularly a sudden fall of the arterial blood pressure and a peripheral vasodilatation.

The heart itself is not appreciably affected, as evidenced by electrocardiograms taken at the height of acute streptomycin poisoning; it continues to beat regularly even after administration of doses which stop the respiration and cause a pronounced fall of the blood pressure (fig. 4). These marked circulatory effects are, however, largely due to the histamine-like contaminant; material which is free from it, as well as pure streptomycin, affects the circulatory system to a far lesser degree. Rabbits under ether anesthesia respond to an intravenous injection of a streptomycin concentrate containing this contaminant with a rise in blood pressure similar to that caused by histamine under such conditions (fig. 5).

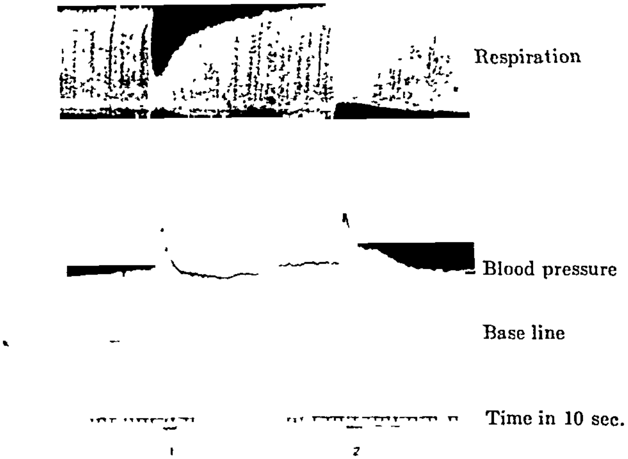

Fig. 5. Similarity of the Effect of a Streptomycin Concentrate and Histamine Hydrochloride on the Blood Pressure of a Rabbit under Ether Anesthesia

1. Histamine hydrochloride 0.025 mgm. per kgm.
2. Streptomycin concentrate 12.5 mgm. per kgm.

Pure streptomycin: Intravenous injection of doses up to approximately 10 mgm. per kgm. of pure streptomycin or an equivalent amount of a material free from the histamine-like factor, has practically no effect upon the blood pressure of the cat (fig. 6). Larger doses of pure streptomycin up to approximately 20 mgm. per kgm. slightly depress the blood pressure, but this effect, in contrast to that of impure material, is rather gradual in onset and recovery and is not accompanied by the striking manifestations of peripheral vasodilation, particularly flushing of the ears and facial and abdominal skin, which are observed after injection of many streptomycin concentrates. Very large doses (120–375 mgm. per kgm.) usually cause irreversible effects. The blood pressure falls to levels of 10–15 mm. Hg. and may remain there for several hours if artificial respiration is maintained. During this period of respiratory and vasomotor paralysis, the heart continues to beat regularly. Death from such excessive doses is probably due to paralysis of the vasomotor centers, which in this stage cease to respond to such central stimuli as increased carbon dioxide tension, metrazol or picrotoxin.

Respiratory effects. The respiratory effect of streptomycin depends upon the dose and the purity of the material. Very small doses, in the order of 0.1–0.2 mgm. per kgm., increase both frequency and amplitude. Larger doses cause respiratory depression, which in non-anesthetized animals is one of the first signs of acute streptomycin poisoning. Thus, non-anesthetized monkeys injected intravenously at a slow rate with 70 mgm. per kgm. of a histamine-free lot of streptomycin in neutral solution, developed severe respiratory distress immediately after the injection. The breathing became slow, irregular and gasping and necessitated the application of artificial respiration in order to carry the animals through the critical period of approximately fifteen minutes. Even these measures, however, failed to prevent the death of one monkey from respir-

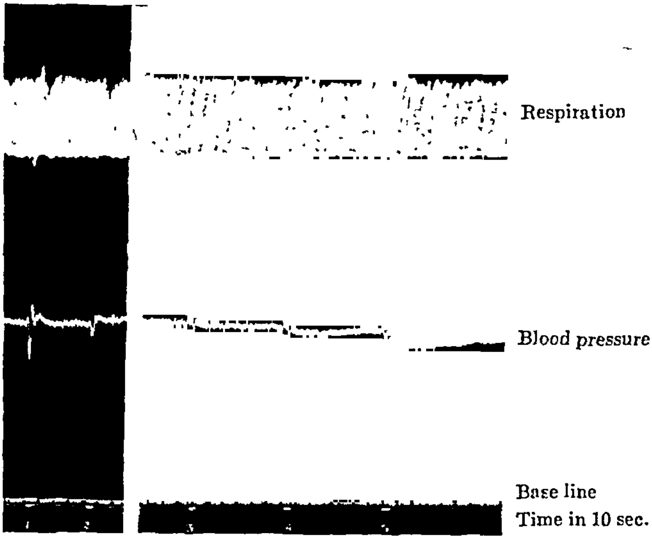

FIG. 6. CIRCULATORY EFFECT OF PURE STREPTOMYCIN IN A CAT (35 mgm. per kgm. Nembutal s.c.)

1. Histamine hydrochloride 0.0001 mgm. per kgm.
2. Histamine hydrochloride 0.00005 mgm. per kgm.
3. Pure streptomycin 5 mgm. per kgm.
4. Pure streptomycin 10 mgm. per kgm.
5. Pure streptomycin 20 mgm. per kgm.

Note the absence of depressor effect with 5 and 10 mgm. per kgm. and the gradual fall with 20 mgm. per kgm., as contrasted to the sharp drop caused by histamine.

atory paralysis. Treatment of a surviving animal with the same total amount of streptomycin, but divided in two doses of 35 mgm. per kgm. two hours apart, greatly reduced the acute respiratory depression. On the other hand, administration by the subcutaneous route of 70 mgm. per kgm. also had an occasional but less severe effect upon the respiration.

Effects on renal function. The effect of streptomycin on renal function was examined in monkeys, dogs, guinea pigs, rats and mice. Two types of experiments were conducted: (a) five hour observation of the water diuresis after administration of a single, relatively large dose of streptomycin; (b) observation of the overnight urine volumes (eighteen hours) during and after a prolonged course of streptomycin administration. The latter type of experiment was considered to be particularly important not only because it more closely resembled the conditions likely to be found in the clinic, but also because temporary impairment of renal function following intravenous therapy with an early lot of streptomycin had been reported (13).

This particular lot, which in man apparently had been responsible for the development of oliguria, proteinuria and hematuria, as well as a large number of other lots of varying potency, were injected into mice and rats over periods ranging from one day to eight weeks. During this time as well as for two weeks thereafter, renal function tests were conducted in the usual manner by measuring the volume and specific gravity of the urine and performing chemical and microscopic urinalyses. The following series of experiments were performed:

In rats: (a) 100 and 200 mgm. per kgm. in single subcutaneous doses; (b) 100 mgm. per kgm. subcutaneously in divided doses daily for five days; (c) 100 mgm. per kgm. subcutaneously daily for eight weeks, with diuresis tests every seventh day.

In mice: (a) 25% and 50% of the L.D. 50 of a streptomycin concentrate (400 and 800 mgm. per kgm.) injected subcutaneously in eight divided doses over a twenty-four hour period; (b) 150 and 300 mgm. per kgm. injected daily subcutaneously in three divided doses for seven days.

In guinea pigs: 30 mgm. per kgm. injected subcutaneously in three divided doses over a twenty-four hour period with daily diuresis tests for the following six days.

All of the above experiments were performed with several lots of streptomycin and were repeated with the same lot in several groups of animals. No indications of renal damage were noted in any of these experiments either intra vitam or at autopsy. The lot which had caused nephrotoxic effects in man failed to affect in a similar manner small experimental animals; the small quantity available prevented its investigation in larger animals which might have been more susceptible, as judged from later experiments with other lots.

As sufficient streptomycin became available, similar studies were performed in monkeys with a large number of lots of varying potency, including pure streptomycin. The following doses were given to a group of 19 monkeys: (a) 25, 50, 100, and 200 mgm. per kgm. subcutaneously in three daily divided doses for five consecutive days; (b) 100 and 200 mgm. per kgm. subcutaneously for ten days; (c) 25, 50 , and 200 mgm. per kgm. intravenously in three or six divided doses for five days.

Renal function experiments were performed during the dosing period and the subsequent observation period. The principal findings were as follows: 1) phenolsulphonphthalein excretion remained unchanged; 2) some of the animals which received the larger doses developed proteinuria; 3) blood urea values increased in almost every animal after the five day dosing period, but remained in the upper normal range; 4) the overnight urine volumes were occasionally decreased; 5) except for 2 monkeys dosed with a low potency material, biochemical findings in the blood remained essentially unchanged. The latter 2 monkeys, however, which were dosed with a material containing only 0.03 mgm. of streptomycin base per mgm. of solids showed a decrease in serum protein level to 4.6 grams per cent but when drug was discontinued, the level returned to normal (7.5 grams per cent) within 2 weeks. Furthermore, on the third day of dosing they showed inflammation at the site of injection and in addition developed edema of the abdominal skin and genital region extending to the upper portion of the legs; fluid withdrawn from the edematous region clotted within a few minutes and the supernatant liquid showed a protein content of 3 per cent.

Intravenous injection of 25 mgm. per kgm. of pure streptomycin or of a streptomycin concentrate free from the histamine-like impurity had no effect on the six hour water diuresis; 200 mgm. per kgm. administered subcutaneously or intravenously caused only a slight decrease in the 18 hour urine volumes. In contrast to this, overnight urine volumes were depressed in animals treated with streptomycin concentrates of average purity and containing the histamine-like factor. In all cases the change followed the same trend, although to a varying degree; it consisted of an initial decrease, most pronounced during the first days of the dosing period, which completely disappeared in the following ten day drug-free observation period. The decreases in overnight urine volumes of 4 monkeys after one day were 75, 18, 67, and 80 per cent respectively; urine excretion was again normal on the tenth day.

The acute effects of streptomycin on water-diuresis were investigated in rats. Three cc. of tap water per 100 grams body weight were administered by stomach tube in the morning and the rats were then kept in groups of five without water and food for an additional three hours, after which they received 5 cc. of tap water per 100 gm. body weight with a dose of pure streptomycin or streptomycin concentrate. The doses employed were in each case 250 and 500 mgm. per kgm. The urinary output was measured at hourly intervals for five consective hours. The results obtained at the 250 mgm. per kgm. level are presented in figure 7.

It can be seen that there was a considerable difference between the effects of pure streptomycin and those of a streptomycin concentrate. The shape of the diuresis curve was only slightly influenced by 250 mgm. per kgm. of pure streptomycin: with 500 mgm. per kgm. the rate of excretion was somewhat retarded, but the five hour total remained approximately the same. In contrast to this, the streptomycin concentrate caused at both dose levels a pronounced retardation of the diuresis and the five hour total urinary excretion was less than one-half of that in the control test. The lot which had caused nephrotoxic effects in man (13) similarly depressed the water diuresis in rats. This inhibitory effect was still noticeable when a control water-diuresis test was performed on the following day, but it disappeared completely on the third day after drug administration (fig. 8).

Effect on smooth muscle. The pharmacodynamic effects of streptomycin on smooth muscle were studied in the isolated rabbit intestine and the isolated guinea pig uterus. Pure streptomycin as well as streptomycin concentrates produced relaxation of both organs when 5–10 mgm. were added to a bath containing 100 cc. of Locke's solution. The effect differed, however, with the type of material used, depending upon the amount of impurities which they contained.

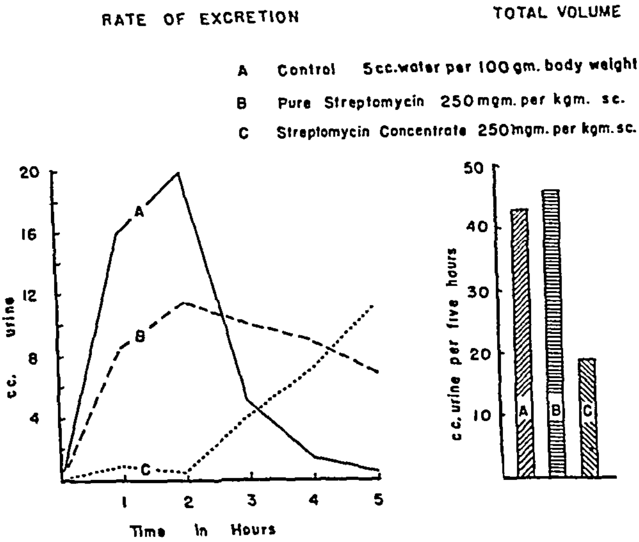

FIG. 7. EFFECT OF PURE STREPTOMYCIN AND STREPTOMYCIN CONCENTRATE ON THE WATER DIURESIS OF RATS
Average of 5 rats

DISCUSSION. The study of approximately three hundred different lots of streptomycin, including the pure compound, reveals great qualitative as well as quantitative differences between materials of equal chemotherapeutic potency but varying purity. These differences greatly interfere with the analysis of the pharmacological and toxicological properties of this new agent, since some properties of the average clinically used material are apparently due to traces of impurities rather than to the active principle *itself*. In this respect streptomycin resembles other antibiotics such as penicillin and streptothricin which vary in their chemotherapeutic, pharmacodynamic and toxicological effects, depending upon the method of preparation. In addition to controllable conditions such as the constancy of the parent microbial strain, the composition of the nutrient medium, culture method, duration of incubation, extraction procedure, and further purification, there always exist certain biological factors which cannot be readily controlled. Prominent among these is the possibility of a natural variation of the culture, especially with regard to its metabolic reaction, which may result in the formation of a product with different chemotherapeutic and pharmacodynamic properties (14). This is well illustrated by the chemotherapeutic differences reported to exist between penicillin X and penicillin G (15, 16, 17) and the variation in toxicity observed in two types of streptothricin produced from different media (9). Since none of the present antibiotics is generally available in the form of a chemically pure compound and even the most careful standardization of production methods cannot give complete assurance that the composition and total amount of impurities in a concentrate will remain constant, it becomes necessary to control each individual lot by biological assays. This is already a pharmacopoeia requirement for penicillin, although this agent has rarely shown toxic effects; it becomes far more necessary for streptomycin which even in its pure form possesses definite toxic properties and in the form of the presently available concentrates is more likely to contain pharmacodynamically active contaminants.

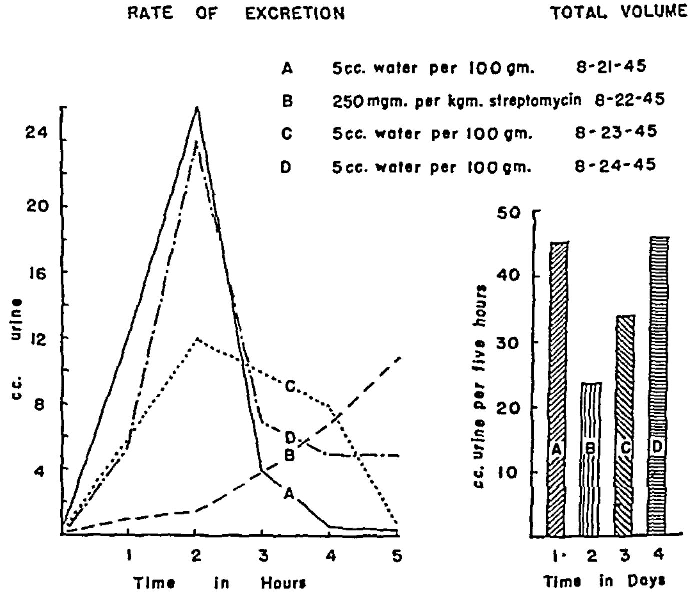

FIG. 8. RECOVERY FROM ANTIDIURETIC EFFECT OF IMPURE STREPTOMYCIN
Average of 5 rats

Our study of the pharmacological and toxicological properties of pure streptomycin as well as streptomycin concentrates has indicated that more than one type of bioassay will be needed, since at least two different types of pharmacodynamically highly active impurities are likely to be encountered.

When administered in sufficiently large doses, pure streptomycin as well as streptomycin concentrates were found to produce death in every animal species examined. The lethal doses varied for the different species but were always far in excess of those needed to obtain therapeutically effective drug concentrations in the blood. The ratio of the L.D. 50 for different routes of administration progressed from approximately 1:10:25 for the intravenous, subcutaneous, and peroral route of administration, respectively. The last figure, however, is of questionable significance, since the amount of streptomycin necessary to produce death by oral administration is so large that unspecific (osmotic) factors are certain to complicate the findings.

The signs observed after administration of lethal doses vary little with the different routes of administration and consist of restlessness, respiratory impairment, loss of balance, unconsciousness, motor paralysis and coma. No characteristic gross anatomical or histological changes were found in animals dying from an acute overdose, except in the case of oral poisoning, where multiple hemmorrhagic lesions in the gastro-intestinal tract were observed. It was found however, that oral administration of sodium chloride in a solution of equal tonicity produced lesions similar to those found in animals fed fatal doses of streptomycin.

The amount of streptomycin necessary to cause death upon parenteral administration was found to have no direct relationship to the potency of the preparation. For example, the subcutaneous toxicity of pure streptomycin in mice was found to be approximately 1500 mgm. per kgm.; a streptomycin concentrate of only one-fourth the potency of this material had approximately the same L.D. 50, while another lot of one-half the potency had an L.D. 50 of 350 mgm. per kgm.; the intravenous L.D. 50 of these three materials was 250 mgm., 125 mgm. and 85 mgm. per kgm., respectively. The rate of absorption from the subcutaneous tissues varies with different streptomycin lots. This can in part explain the variation in the subcutaneous toxicity of lots of equal potency. It can, however, not account for the differences found after intravenous injection. We must assume, therefore, that an impurity not revealed in the microbiological assay is responsible for differences in the intravenous toxicity of lots of thesame potency. This is further indicated by the fact that of twenty-eight different lots examined for intravenous toxicity (fig. 3) none was found to be less toxic than pure streptomycin, while twenty-six exceeded its toxicity up to four times.

Great variations were also noted in the morbid toxicity of streptomycin, particularly in the effect upon the blood pressure. Pure streptomycin in doses up to 10 mgm. per kgm. has practically no effect upon the blood pressure of an anesthetized cat; larger doses produce a gradual fall, followed by an equally gradual recovery; and only large doses (75 mgm. per kgm. and more) depress the blood pressure to very low levels.

Some lots of streptomycin concentrates are as free from this depressor effect as pure streptomycin itself; the majority, however, cause a pronounced fall of the arterial blood pressure, which in contrast to that produced by excessive doses of pure streptomycin is quite sudden and closely resembles that produced by an intravenous injection of histamine. In cats of equal sensitivity to histamine the doses of streptomycin concentrates necessary to lower the blood pressure vary from 0.005 mgm. to 2 mgm. per kgm., a futher indication that this depressor effect is due to impurities and not to streptomycin itself. There is, however, no close correlation between the degree to which individual lots produce the depressor effect and their intravenous and subcutaneous lethal toxicity. We assume therefore that at least two different contaminants may frequently be present in streptomycin concentrates. The exact chemical nature of these impurities is not yet known; we have, however, been able to remove the one causing the vasodepressor action by incubation with histaminase[4] and the conclusion seems therefore justified that it may be related to histamine.

TABLE 1

Lethal toxicity of highly purified streptomycin samples

SAMPLE NUMBER	POTENCY, MICROGRAMS STREPTOMYCIN BASE PER MGM.	BLOOD PRESSURE (HISTAMINE-LIKE) EFFECT	L.D. 50, MGM. PER KGM.		RATIO OF INTRAVENOUS TO SUBCUTANEOUS L.D. 50
			Intravenous	Subcutaneous	
9327	800	negative	75	300	1:4
9271	800	negative	100	600	1:6
320	800	negative	145	600	1:4+
8798	670	negative	150	750	1:5
225	700	negative	170	850	1:5
389	800	negative	187	750	1:4
9803	750	negative	250	1250	1:5
9767	750	negative	300	1150	1:4−

Such treatment of a streptomycin concentrate containing the histamine-like factor also decreased the inhibitory effect on water diuresis in rats. It was, however, without effect on the subcutaneous and intravenous toxicity in mice. A further indication that the histamine-like factor is not the only one influencing the toxicity may be seen from the data presented in table 1. This shows the acute L.D. 50 upon intravenous and subcutaneous administration of eight highly purified streptomycin samples which had approximately the same potency (670–800 micrograms streptomycin base per mgm.) and were free from the histamine-like contaminant. While the ratio between the lethal doses after intravenous and subcutaneous administration remained relatively constant for any given sample, a four-fold difference in the magnitude of the lethal dose existed between the least and the most toxic sample.

While the pharmacodynamic effects of large parenteral doses of streptomycin are quite marked, they are nevertheless not likely to have great practical implications. In the mouse, the ratio between a chemotherapeutically effective and a lethal dose is from 1 to 100 upward. Other species, such as dogs, cats and monkeys, are known to be more susceptible to the pharmacodynamic effects of streptomycin or its contaminants; nevertheless, the nature of these acute side-effects is such that it is most unlikely that they could create a serious hazard in the clinical use of this drug. It is more important to have information on the chronic toxicity of streptomycin since patients suffering from tuberculosis, brucellosis or similar chronic infections may require its administration for a long time.

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[4] We are indebted to Dr. M. L. Tainter of the Winthrop Chemical Company for supplying us with histaminase.

Streptomycin is toxicologically not as inert as penicillin, but it nevertheless appears to be one of the least toxic chemotherapeutic agents. Rats, mice and guinea pigs treated for several months with large doses showed no significant pathological changes. Experiments in monkeys and dogs, however, produced definite signs of toxicity, consisting of occasional retention of bromsulphalein, a transient anemia, mild to severe proteinuria and the appearance of casts and blood cells in the urine. Dogs also showed signs of impaired vestibular function and, possibly decreased auditory acuity. Autopsy revealed a fatty metamorphosis in the liver and kidney. However, monkeys dosed for varying lengths of time failed to exhibit a progressive increase in the pathologic changes and were found to return to normal after discontinuation of the treatment. Furthermore, the great difference in species susceptibility leaves open the possibility that man may not react in the same manner to large doses of streptomycin. However, until more is known regarding this point, it would seem advisable to pay close attention to the renal and hepatic function of patients treated for a prolonged time with large doses of streptomycin. In this connection, it may be of interest to note, that the laboratory diet of rats which completely fail to develop pathologic changes from prolonged administration of streptomycin, is relatively high in protein, whereas monkeys which were found to be highly susceptible, received a diet relatively low in protein. Experiments for the purpose of clarifying the possible influence of these factors, are now under way. It may be appropriate, however, to suggest at this time that patients under prolonged streptomycin therapy should be kept on a high protein intake.

In evaluating the toxic effects of streptomycin a distinction must be made between those caused by the pure compound and those due to frequently present impurities. Of these, one of the pharmacodynamically most active is of histamine-like nature. It is responsible for the fall of blood pressure in those species which respond in a like manner to histamine and is probably also a contributory cause of the acute inhibition of the water diuresis, since histamine produces a similar effect (18, 19). Since the amount of the histamine-like factor found in an average streptomycin concentrate is equivalent to approximately 0.4 mgm. of histamine hydrochloride per gram of streptomycin base and may occasionally go as high as 10 mgm., many of the pharmacologic effects of streptomycin concentrates can obviously be ascribed to this impurity. In man, histamine doses of 0.5 mgm. subcutaneously and 0.02–0.05 mgm. intravenously are known to produce pronounced pharmacodynamic effects (20, 21). These are, however, of relatively short duration and do not appear to constitute a serious threat to life. It may be appropriate, however, to point out, that Dale (22) found that animals which had been subjected to a prolonged ether anesthesia became much more sensitive to the circulatory effects of histamine. It would appear, therefore, that special caution should be exercised in the administration of streptomycin to patients during, or several hours after, a prolonged ether anesthesia.

It is unlikely that the histamine-like impurity is responsible for the manifestations of chronic toxicity, exhibited particularly in the liver and kidney. While many of the biochemical and pathological changes found in dogs and monkeys after large doses of streptomycin resemble those occurring in animals subjected to histamine shock (23, 24), the doses of histamine used in these experiments exceeded the quantity of the histamine-like substance present in our streptomycin concentrates. Furthermore, prolonged administration of pure streptomycin was found to produce in monkeys the same pathologic changes as streptomycin concentrates.

SUMMARY

1. The pharmacological and toxicological properties of streptomycin were found to be greatly influenced by the presence of traces of impurities and varied considerably in different animal species. One such impurity has histamine-like properties and can be inactivated by histaminase.

2. The signs observed after administration of large doses of streptomycin consist, in order of appearance, of restlessness, respiratory distress, loss of consciousness, coma and respiratory failure. The latter is apparently the cause of death, since the heart continues to beat for several minutes after cessation of the respiration.

3. The acute intravenous toxicity of streptomycin increases greatly with the speed of injection. This factor is less influential with the intramuscular and subcutaneous administration. The rate of absorption varies with different lots and influences and outcome of subcutaneous and intramuscular toxicity experiments.

4. No pathologic changes were found in the organs and tissues of animals killed in acute toxicity experiments.

5. The signs and symptoms produced by repeated daily administration of streptomycin over periods of 5–80 days vary greatly with the animal species. Weanling rats show a slight retardation of growth and develop a pronounced nervous hyperexcitability. No such changes were noted in adult rats or in mice. Monkeys exhibit a slight impairment of hepatic and renal function and show upon autopsy a fatty infiltration of the liver and, to a lesser degree, of the kidney. These changes are reversible. Dogs develop a mild to severe impairment of renal function, and show upon autopsy lipoid deposits in the kidney and liver. In addition, they exhibit signs of cerebellar or labyrinthine disturbance.

6. The injection or topical application of streptomycin usually does not produce marked local effects.

7. Intravenous injection of streptomycin concentrates is often followed by a sharp drop of arterial blood pressure due to a histamine-like impurity. Pure

streptomycin is free from this effect, but produces in large doses a gradual and prolonged depression of the blood pressure.

8. Injection of a large dose of streptomycin concentrate may cause a marked temporary inhibition of water diuresis. No such effect is found with pure streptomycin.
9. Streptomycin causes a relaxation of the isolated intestine.

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THE ANTAGONISM BETWEEN ATROPINE AND STRYCHNINE IN THE MOUSE

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According to Calma and Wright (1) atropine counteracts the effects of acetylcholine in the central nervous system, and Marazzi (2) has shown that it depresses transmission in sympathetic ganglia. When large doses are injected into animals depression occurs which may last 24 hours or more and this may be followed by convulsions (3). In man the excitatory phase usually appears first followed by depression. In dogs atropine acts synergistically with barbiturates (3). The depressant phase which is primarily central may be due to the impairment of transmission in the brain and spinal cord and consequently atropine should conteract some of the excitatory effects of cholinesterase inhibitors. The conditions necessary to obtain such an antagonism would be such that the effects of the esterase inhibitor wear off during the depressant phase of atropine i.e. before atropine itself acts as an excitant. Such conditions were apparently not obtained by Koppanyi (3) who found that atropine and strychnine were synergistic in the dog. In the mouse, however, it has been shown (4) that atropine protects against an MLD_{100} of strychnine, and the present work is an extension of this observation. Strychnine is the best drug to use because its central effects are almost entirely excitatory and this may possibly be attributed to its inhibition of the cholinesterase (5).

EXPERIMENTAL. Female and male white mice weighing 20–25 gms. were used. The MLD_{100} of strychnine was determined for each experiment and was found to be constant at 1.33 mgm./kgm. Atropine was given in a dosage of 400 mgm./kgm. Both drugs were injected subcutaneously. Figure 1 summarizes the results. When strychnine and atropine are injected simultaneously almost 60% of the animals recover. As the time between the injection of atropine and strychnine increases there is a definite increase in the percentage of recoveries which reaches a maximun when strychnine is injected one hour after atropine. This would indicate that the concentration of atropine rises to a maximun in the central nervous system about one hour after a subcutaneous injection. As the interval between the two drugs is increased the percentage of recoveries falls steeply to about 50%, possibly because a further redistribution of atropine occurs and some is lost by the central nervous system. After this, the percentage recoveries decrease at a uniform rate which indicates that the amount of atropine excreted at any time is proportional to the amount in the body and that neither the kidney nor any organ has any special mechanism for dealing with the drug. These facts can be formulated in the following equations. If x = amount of atropine in the body and t = time and c = constant, then $dx/dt = -cx$ which when integrated gives $\log x = -ct + A$. The effect of atropine, in this case measured by the percentage of recoveries, R, is equal to $k \log x$. Substituting for $\log x$, $R = -ckt + AK$, or grouping the constants, $R = B - Ct$. The values for B and C calculated by the method of least squares, which fit the experimental points are given in the legend of figure 1.

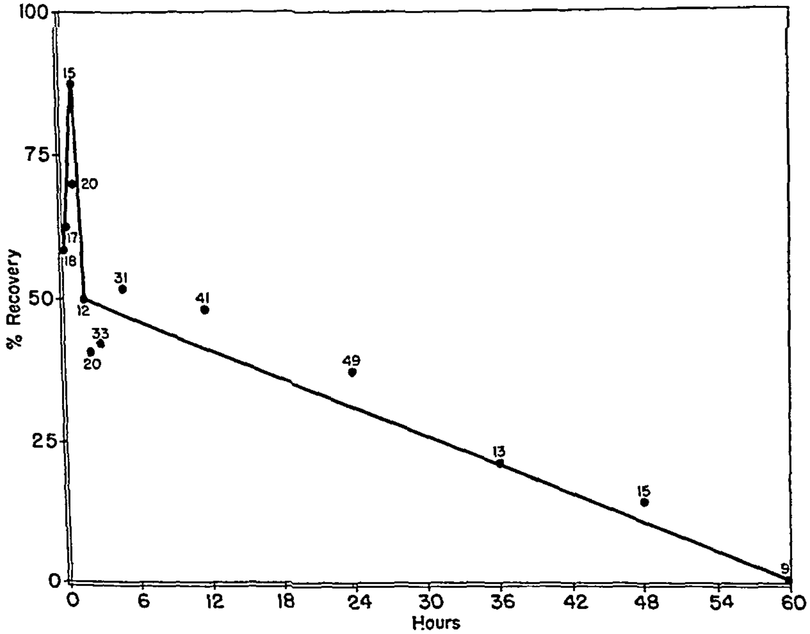

FIG. 1. THE EFFECT OF PREVIOUSLY ADMINISTERED ATROPINE ON THE PERCENTAGE RECOVERIES OF MICE INJECTED WITH MLD_{100} OF STRYCHNINE PLOTTED AGAINST THE TIME INTERVAL BETWEEN THE INJECTION OF THE TWO DRUGS

The points represent the experimental values and the figures the number of mice used to establish them. The straight line is drawn from the equation $R = 52.4 - 0.85t$ where R = % recovery and t = time in hours between the injection of atropine and strychnine.

The interval between the injection of strychnine alone and the time of death averaged 5 minutes. When deaths occurred after atropine the time interval was always prolonged, varying from 25 minutes when strychnine was injected 2 hours after atropine to 6 minutes when injected 24 hours after atropine. Homatropine in a dose of 200 mgm./kgm. is also an effective antagonist to strychnine.

DISCUSSION. There is no evidence that strychnine *in vivo* has any important peripheral action. Atropine, of course, has a large number of peripheral loci of action but these are primarily at autonomic endings and ganglia. Large doses of atropine do depress transmission at the myoneural junction of skeletal muscle but it is doubtful whether this can account for its protective action against strychnine. If atropine is known to antagonize the central action of acetylcholine, if strychnine inhibits the cholinesterase, and if acetylcholine accumulates in the frog brain during the strychnine convulsions (6, 7) it is reasonable to postulate that the antagonistic effects of the two drugs occurs in the central nervous system.

SUMMARY

1. Atropine in a dosage of 400 mgm./kgm. protects mice against 1.33 mgm./kgm. of strychnine.

2. The percentage of recoveries varies with the time interval between the injection of atropine and strychnine. It is maximal when this interval is one hour. Some protection is however obtained after an interval of 48 hours.

3. The significance of this is discussed.

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ELECTROCARDIOGRAPHIC CORRELATIONS WITH DIGITALIS BIOASSAY

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Numerous investigations have been made on the electrocardiographic effects of intravenous digitalis in the experimental animal. One of the earlier reports (1) dealt with the electrocardiographic changes brought about when one-tenth of the estimated lethal dose was injected at ten minute intervals. The investigators also studied the effect of bilateral vagotomy. Gold et al. (2) compared the electrocardiographic changes of seven varieties of digitalis when given intravenously at five minute intervals in the cat. Qualitative changes were similar in each case but individual variations were extreme. These investigators could find no electrocardiographic sign which was as accurate as the minimum lethal dose in evaluating the toxicity of the drug (3). Their experiments were performed on the cat without the use of a general anesthetic. The electrocardiographic changes have also been studied (4) when the intravenous injection was continuous and maintained at a constant rate by means of a pump. It was found that vagus effects under these conditions could not be abolished by atropine when the glycoside digitoxin was employed. There is wide disagreement in these reports on the quantitative relationship of electrocardiographic signs to the minimum lethal dose, to the difference in effect of various digitalis preparations and to the effect of rate of injection on the minimum lethal dose. The present report deals with some of these factors by studying a larger number of cases by essentially the same methods as have been used previously.

METHODS. Electrocardiographic studies were made during the bioassay of digitalis by the USP XII method (5). The procedure requires an intravenous injection of 1 cc. per kgm. at five minute intervals, of a dilution of the unknown sample estimated to kill the animal in 15 doses. The test animal used is the cat maintained in light ether anesthesia. If the preliminary cat requires more than 19 or less than 13 doses, the dilution is modified in subsequent animals to produce death at about the middle of this range. The strength of the preparation is calculated in terms of the minimum lethal dose of Standard Reference Digitalis, which is assayed by the same procedure. It is important to note that the prescribed method reduces tinctures of an unknown strength to a common value. By arbitrary dilution a point is reached at which each tincture gives a mean value of 15 or 16 doses. Hence, variation in the number of doses producing death, regardless of the sample involved, is essentially a deviation from a common mean.

All electrocardiograms were taken with the animal under ether anesthesia. One or two control records were obtained before commencing the injections. Subsequently, a record was obtained a few seconds before each injection until the death of the animal. Extra electrocardiograms were taken when changes in the string could be detected visually. All three standard leads were occasionally taken, but only lead II routinely. With the animal tied on its back in the position for injections, axis deviation renders the excursions in lead I almost negligible. One control experiment was performed in which normal saline containing the same strength of alcohol as the digitalis dilutions, was injected at five minute intervals for a period of two hours. As a regular control one or two electrocardiograms were taken on each cat before commencing the digitalis injections. Two hundred and fifty cats were studied in this manner. In 34 animals the femoral arterial blood pressure was recorded throughout the experiment, in most cases by a mercury manometer. In 11 animals the right or the left vagus nerve was sectioned before injections.

Results. The electrocardiographic features of 218 cats have been studied in some detail. This number was determined by eliminating those in which special procedures had been employed, those in which a non-digitalis death was suspected and a few cases in which the electrocardiographic records were faulty or incomplete.

Coupling. Table 1 lists the incidence of various electrocardiographic features in the records taken before and after the administration of digitalis. Of particular interest is the large number of cases showing coupling (a ventricular premature alternating with a normal cycle) before administration of digitalis. Kurtz, Bennet and Shapiro (6) reported extra systoles in 20% of human cases in the operating room when ether was the anesthetic employed. Frommel (7) had previously reported the occurrence of coupling in guinea pigs under ether anesthesia. In our cases coupling usually disappeared after the animal had been under ether for a sufficient period of time and had reached a steady stage of surgical anesthesia. The observation suggests that the induction rather than the maintenance of anesthesia brought on the reaction. The incidence of coupling in the control records was not closely correlated with either the A-V dissociation dose or the lethal dose; however, the animals having a small lethal dose tended to show a hight percentage of coupling in the pre-digitalis records. As a further check on the anesthesia involved, the standard three leads were taken on 67 cats that were being prepared for student laboratory use. Most of these were given 30 mgm. per kgm. of nembutal intraperitoneally, a few 35 or 40 mgm. per kgm. None of these exhibited coupling. This arrythmia is commonly assumed to be a characteristic symptom of digitalis overdosage (8). However, under the conditions of these experiments its occurrence during the administration of digitalis was infrequent (9%).

TABLE 1

Incidence of ECG events up to the time of A-V dissociation

% INCIDENCE	COUPLING	P-R >.08	NODAL RHYTHM	T WAVE NO CHANGE	T WAVE ABSENT OR INVERTED	S-T DEVIATION	
						Pos.	Neg.
% Before digitalis	25	17	0.9	15	29	4.0	3.0
% After digitalis	8.7	42	14.2	15	56	15	56

P-R interval. A P-R interval of 0.08 sec. occurred most frequently in both the predigitalis and the digitalis records. Values greater than this were more frequent during the administration of the drug. In figure 1 are shown the average heart rates plotted against the P-R interval for control records, and for the digitalis records at a point just before A-V dissociation occurred. An inverse relationship is found in both instances. The significant difference of the points forming the two lines has been calculated and according to the distribution of Student's t values (9) the probability of the means being due to chance ranges from 0.09 in 100 to 2.9 in 100. It is surprising to find that the P-R interval for given rate is always smaller in the case of digitalis. If one accepts the usual explanation that vagal activity accounts for the inverse relationship between heart rate and the P-R interval, it would seem to imply that digitalis at this stage had reduced the heart rate primarily by direct action on the SA node. Certainly, there is no evidence for a direct depressant action on A-V conduction, a fact which harmonizes with the observation that A-V dissociation (rather than A-V block) is the succeeding event in the course of administration of the drug. In the reports referred to above (1), (3) a prolongation of the P-R interval is offered as evidence of the action of digitalis and yet an examination of the accompanying heart rates shows that similar rates in the absence of digitalis (figure 1) are associated with P-R intervals of equal or even greater magnitude. *T Wave.* It will be noted in table 1 that the absence or inversion of T waves frequently occurs before digitalis, but 15% of the cases showed no T wave changes up to the point of dissociation. An examination of the remaining cases in which changes of the T wave follow the administration of the drug, shows wide variation as to the amount of drug necessary to produce the effect. This failure to find T wave changes quantitatively related to dosage, disagrees with the work of Robinson

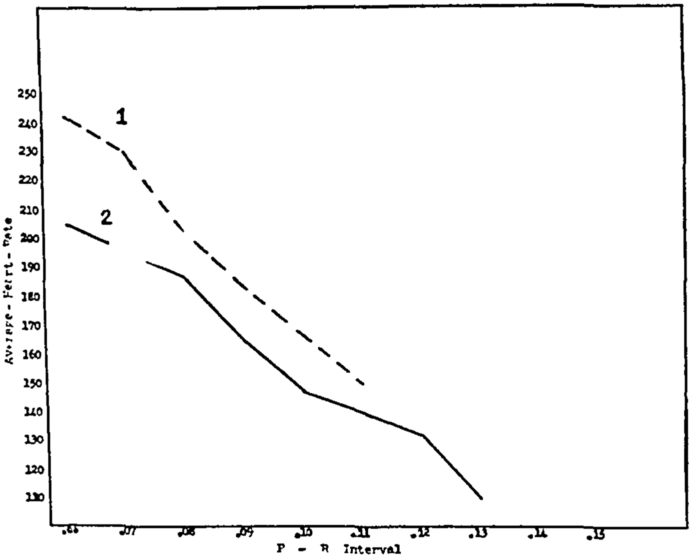

FIG. 1. Line 1 represents values obtained in control records with the animal under ether. No digitalis had been given.

Line 2 represents values obtained during the administration of digitalis. The values usually represent the last record before the occurrence of A-V dissociation.

and Wilson, but agrees with the results of Gold et al. (2) who used cats under local anesthesia. The unreliability of the T wave changes as a quantitative index has also been reported when animals other than cats were used (10), (11). Such results are fully to be expected if the form of the T wave is considered in terms of the ventricular gradient. The effect of ouabain upon the latter factor has been reported recently (12).

In figure 2 the cases are grouped according to the dose producing A-V dissociation and then the average dose at which each of the events occurred is plotted against the group. The approximate order of events from the beginning of the injection to the death of the animal is: maximum $\frac{R\text{-}R}{Q\text{-}T}$ ratio, minimum sinus rate, S-T segment deviation (usually negative), ectopic beats (ventricular or nodal), A-V dissociation, paroxysmal tachycardia (from one or more ectopic foci), ventricular fibrillation and cardiac death.

$\frac{R\text{-}R}{Q\text{-}T}$ *ratio.* Due to the fact that a heart rate of about three times the human values was being recorded at standard paper speed it was not possible to calculate the Q-T constant in terms of the cycle as has been done by Bazett (13) and others (14). However, $\frac{R\text{-}R}{Q\text{-}T}$ values based on an average of several cycles in each record did show a progressive increase up to a point slightly preceding the minimum rate.

S-T segment deviation. The S-T segment deviation preceding dissociation was usually depressed and was a more constant phenomenon than changes in the T wave alone. Occasionally, there was a slight but consistent elevation. In a few special experiments these changes were approximated by applying a diluted tincture of digitalis to the external surface of the ventricle, confirming the results obtained with KCL solutions which were reported by Hoff, Nahum, et al. (15). The application of digitalis to the right ventricle depressed the S-T segment, on the left ventricle elevation occurred, whereas mixed results were obtained in the three leads with application to the posterior or anterior surfaces. This is in complete agreement with the report of the workers who used KCl solution. Identical results also were obtained with a watery effusion of digitalis thus eliminating the possibility of alcohol being the effective agent. The authors referred to above have assumed that the electrocardiogram is produced by activity at the surface of the heart and that KCl solution applied to the surface abolishes such electrical activity at the point of application. Thus, by applying KCl to one ventricle or the other, they have been able to record the dextro or the laevocardiogram and to construct from them, by algebraic summation, the normal QRS and T waves. This interpretation, however, does not explain the fact that we obtained a profound depression of the S-T segment when the entire surface of both ventricles was exposed to either KCl solution or to digitalis.

Ectopic beats. These occasionally preceded the point of A-V dissociation but usually they were first recorded at the time of dissociation. Hence, the average dose at which they appeared as shown in figure 2 closely approximates a straight line relationship.

Ventricular fibrillation. This was usually the terminal event. In a few cases cardiac death was preceded by chaotic rhythms that could not be classified as ventricular fibrillation. In some instances ventricular fibrillation lasted but a few seconds and another injection was given before death occurred. This accounts for the ventricular fibrillation line slightly preceding the line representing the minimum lethal dose in figure 2. In a number of cases there was progressive deepening of the depressed S-T segment which eventually obliterated the QRS complex and merged into a smooth wave typical of ventricular fibrillation.

A-V dissociation. This term is preferable to A-V block as the electrocardiographic evidence indicates that the independent ventricular rhythm at this point is due to excessive stimulation of the ventricles rather than to deprivation of the normal impulses from the SA node. At some point during the administration of the drug complete and permanent A-V dissociation occurs. Occasionally, it is initiated as a middle or lower nodal rhythm but usually the first electrocardiogram shows a rapid-ventricular ectopic rhythm with the auricular rate likewise increased. Infrequently dissociation is followed by a short period of sinus rhythm before becoming permanent. The actual events immediately preceding the first record of dissociation can only be inferred statistically as dissociation in any given case may have occurred at any moment during the five minute period between injections. There is no apparent simple relationship with preceding

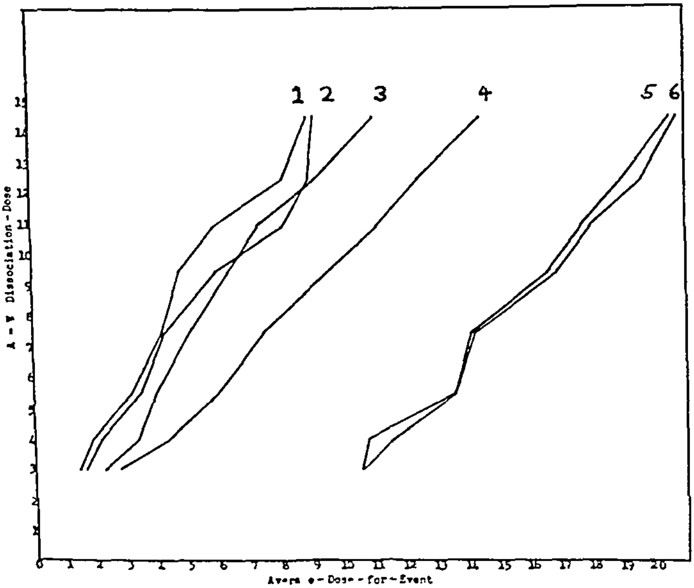

FIG. 2. Cases are grouped according to A-V dissociation dose. 1—maximum $\frac{\text{R-R}}{\text{Q-T}}$; 2—minimum rate; 3—S-T Segment Deviation; 4—Ectopic Beats; 5—Ventricular Fibrillation; 6—Lethal Dose.

events. The sinus rate usually but not invariably reaches a minimum just preceding A-V dissociation. In order for the transition from a sinus rhythm to A-V dissociation to occur it must be presumed that the A-V node or bundle has a rate of impulse formation nearly equal to or greater than, that of the SA node. If the cases are grouped according to the $\frac{\text{Post A-V dissociation rate}}{\text{Pre A-V dissociation rate}}$ the distribution curve shows the peak value at a ratio of one. Pre A-V dissociation rates tend to reach a peak just preceding this point and then progressively decline, suggesting that the ratio is dependent within limits upon the sinus rate (figure 3).

Characteristically both the auricular and the ventricular rates continue at a very high level until the onset of fibrillation. The auricular rate may vary considerably during this period and occasionally the auricles continue beating for a time after ventricular standstill has occured. The ventricular ectopic focus as judged by the direction of the initial complex may be in either the right or the left ventricle. Before the onset of fibrillation there is frequently evidence of multiple foci. A-V dissociation has been reported to occur from 50% to 80% of the minimum lethal dose (16), (17). Some of the variations are undoubtedly due to differences in anesthesia, rate of infusion, etc., but in most reports there is considerable variation within a given group of cases subjected to the same conditions.

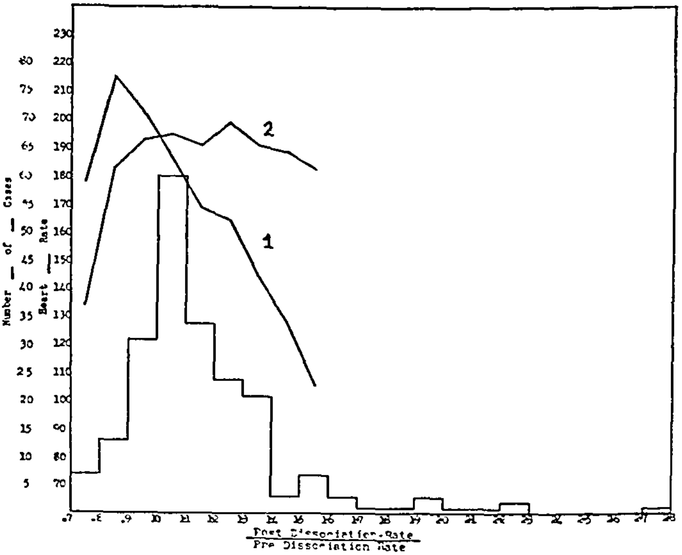

FIG. 3. 1—Pre-dissociation heart rate; 2—Post-dissociation ventricular rate.

If the cases reported here are grouped in terms of lethal dose and the average A-V dissociation dose is plotted against the lethal dose, the former occurs at about 50% of the lethal dose. On the other hand, if the cases are grouped according to the dose at which A-V dissociation occurs it can be seen that the average minimum lethal dose tends to be the same for some of the adjacent A-V dissociation groups (table 2). This is probably due to the admitted error in minimum lethal dose estimates by the present method (18). It seems significant, however, that when the A-V dissociation dose is plotted against the average lethal dose by this grouping, there is a strong suggestion that the latter occurs at a constant dose or time interval after the advent of dissociation, and not on a percentage basis. This relationship is shown in figure 4. A line so drawn that the A-V dissociation dose plus 7 doses equals the lethal dose, agrees quite well with the distribution of points and all of the points fall well within an area representing twice the average S. E. The significant feature is the fact that a constant number of doses or time, intervenes between dissociation and death, even though the lethal dose varies 100% or more. This would imply that the chief variable is the point of complete A-V dissociation and that the resistance to a toxic level of digitalis beyond this point is fairly constant from one individual case to another. The significance of dissociation as given here is of course valid only if the phenomenon is the result of a progressive increase in digitalis. In two cases, dissociation due to other causes was present before any digitalis had been given and in both instances the minimum lethal dose was approximately the same as the average for the series.

TABLE 2

Average minimum lethal dose of cases grouped according to dose which produced A-V dissociation

A-V DISSOC. DOSE	3	4	5	6	7	8	9	10	11	12	13	14	15
Average M.L.D.	10.6	11.6	13.7	13.6	14.2	14.3	16.9	17.0	18.0	19.2	20.0	20.7	20.7
No. of cases	8	19	29	24	17	22	20	17	17	9	17	4	4

Blood pressure. A few preliminary observations on blood pressure during the bioassays seemed to indicate an inverse relationship between this value and the minimum lethal dose. On the assumption that this relationship might be due to rate of coronary flow, continuous blood pressure was recorded by a mercury manometer from the femoral artery in 34 cases. No significant relationship between mean blood pressure and lethal dose, A-V dissociation or other electrocardiographic findings could be demonstrated. With a few exceptions the rate of fall of mean blood pressure from the beginning to ventricular fibrillation was fairly constant and at an approximate value of 4 mm. Hg per dose or per five minute interval. Complete A-V dissociation did not hasten the rate of fall.

Vagotomy. The right vagus was sectioned in four animals in a series of nine cats. The control rate and rate immediately before dissociation were much faster in the vagotomized animals but the decrease in rate was small. The minimal rate appeared much earlier during the administration of the drug. Likewise, both the average A-V dissociation dose and the lethal dose were smaller. In only one of these animals was there a significant increase in P-R interval.

In a series of 10 animals the left vagus was sectioned in 7 cases. As in the case of the previous group, the heart rate immediately preceding dissociation was faster in the vagotomized animals and only one case showed a significant increase in the P-R interval. However, the average dose for the minimal rate, dissociation and death did not vary appreciably from the control group.

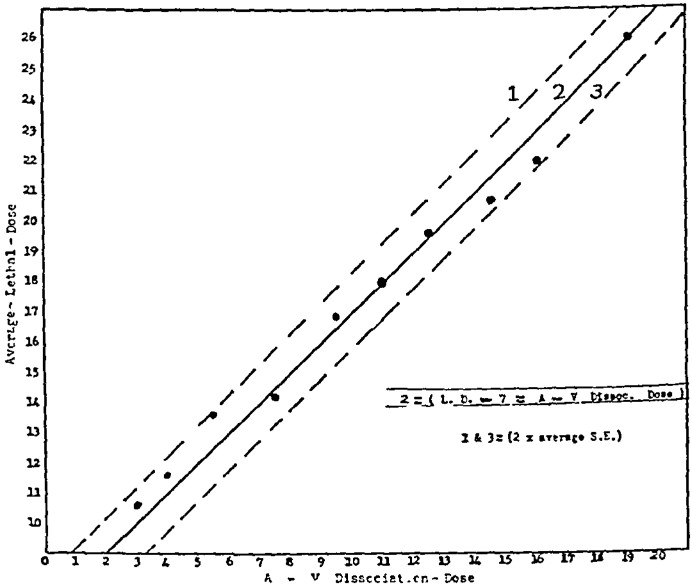

FIG. 4. The Standard Error of the average L. D. ranged from 1.0 to 0.34. The average S. E. equaled 0.62. Broken lines 1 and 3 represent twice the average S. E. The solid line 2 is arbitrarily drawn by adding 7 to each A-V dissociation dose.

TABLE 3

Average results from cutting right or left vagus nerve

TYPE OF CASE	NO. OF CASES	A-V DISSOC. DOSE	LETHAL DOSE	CONTROL RATE	DOSE OF MIN. RATE	RATE PRECEDING A-V DISSOCIATION
R. Vagus	4	7.7	13.0	225	4.7	216
Control	5	11.0	17.0	161	8.8	120
L. Vagus	7	10.0	16.3	206	7.0	194
Control	4	9.0	19.0	200	5.0	125

The results are based on too small a number of cases to have statistical significance. The fact that the P-R interval rarely increases with vagotomy suggests the importance of reflex regulation of the rate of conduction in the A-V node. Robinson and Wilson obtained similar results with bilateral vagotomy in respect to changes in heart rate and A-V conduction. Dissociation occured earlier in only one of their cases. Our results might be taken as an indication that the vagal effects of digitalis reside primarily in the right vagus. The results are summarized in table 3.

SUMMARY

A statistical study of electrocardiographic changes during the bioassay of digitalis by the USP XII method indicates the following:

(1) Under ether anesthesia coupling is more frequent before than after the infusion of digitalis.

(2) The P-R interval for a given heart rate is smaller under the influence of digitalis.

(3) The average dose at which the maximum $\frac{R\text{-}R}{Q\text{-}T}$ ratio, minimum rate, S-T segment deviation, ectopic beats, ventricular fibrillation and cardiac death occur, varies with the dose producing A-V dissociation.

(4) When the cases are grouped according to the dose producing A-V dissociation, the interval between this event and the lethal dose tends to be a constant one.

(5) Mean blood pressure bears no significant relationship to the electrocardiographic findings.

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A STUDY ON THE DEVELOPMENT OF TOLERANCE AND CROSS TOLERANCE TO BARBITURATES IN EXPERIMENTAL ANIMALS

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Tolerance for many drugs can be acquired by human beings as well as by experimental animals. When this occurs the dose of the drug has to be progressively increased in order to maintain a given level of pharmacological effect. In dealing with the question of the possible formation of a tolerance to the barbiturates there has been much confusion and contradiction among various investigators. This lack of agreement can be attributed to several factors: (a) the loose usage of the terms "drug tolerance" and "drug addiction" or "habituation," (b) differences in the time intervals between repeated injections of the drug, (c) differences in the quantity injected per dose, (d) differences in the criteria used in determining acquired tolerance, (e) differences in the species of animals used by the various investigators. In most instances the reports have been based upon the results of experimentation on one or at most six animals.

Deuel, Chambers, and Milhorat (1) studied the influence of amytal upon the metabolism of the dog. They concluded that the dog does not acquire an increased tolerance for amytal since the same dose always produced the same effect. Their conclusion was based primarily upon the results from one animal in which 20 different injections were made from time to time over a period of approximately 10 months. They, however, do not specify how long a time had elapsed between each injection and the next.

Their results have been confirmed by Swanson, Weaver, and Chen (2). A group of 10 dogs and 6 monkeys were given sodium amytal. They state that they found no evidence of tolerance since the same dose, repeatedly employed, was always effective and never failed to produce hypnosis. They also observed no decrease in the toxicity of the drug since the LD_{50} killed 5 of the 10 experimental dogs. Since no change in the speed of induction of sleep was noted in rats following repeated administrations of amytal, Ravdin, Drabkin and Bothe (3) concluded that tolerance to this drug cannot be acquired.

On the other hand lethal doses of amytal were given by stomach tube to groups of 2 to 4 rabbits by Fitch (4). In all instances the duration of sleep was shortened by 50 per cent on repeated administrations and the dose tolerated was much larger than the LD_{50} for non-tolerant animals of the same species.

Using rabbits, Masuda, Budde, and Dille (5) were able to show a progressive decrease in the duration of sleep following repeated administrations of amytal. This acquired tolerance to the drug disappeared rapidly after ending the daily injections. Within 3 or 4 days the animals responded to the barbiturate in practically the same way as they had done to the first dose of the drug.

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Rats given repeated subcutaneous injections of sodium amytal develop a high degree of resistance to the drug according to Nicholas and Barrow (6). Following such a procedure they observed 2 male and 6 female animals to require twice the standard dose of the drug to produce deep anesthesia.

Mott, Woodhouse, and Pickworth (7) studied the effects of veronal, luminal and dial on cats and monkeys. Although the results were by no means conclusive they remark that when the doses were slowly increased the animals seemed to acquire some degree of tolerance to moderate doses of the drugs.

Four dogs were used by Oettel and Krautwald (9) in their investigations. Upon repeated administrations of the same dose of veronal they observed a progressive decrease in the duration of sleep. A similar shortening of the sleeping times were noted, when luminal, phanodorn, or noctal were similarly administered. However, reports published by Bachem (10) and by Hofmann (11) each based on observations on one animal, gave negative results on the establishment of a tolerance to veronal.

Following the administration of repeated doses of sodium barbital, Seevers and Tatum (24) observed a shortening of the sleeping time in all six dogs so studied. However, at no time, did the animals reach a stage where the drug failed to produce depression and sleep. Hoff and Kauders (25) report an increase in the MLD in one dog by subacute poisoning of two months duration. This observation was not confirmed by Seevers and Tatum.

Repeated intravenous injections of dial were made in seven dogs by Ettinger (13). In three animals the first injection produced anesthesia lasting for at least 8 hours, while later injections of the same amount caused anesthesia of an hour or less in duration.

Eddy (8) was unable to show the development of increased tolerance in 4 cats to daily oral administrations of barbital. After two weeks treatment the sleeping time was practically the same as had been induced when the drug was given for the first time.

Lethal doses of neonal and noctal were given to groups of 2 to 4 rabbits by Fitch (4). With these drugs as with amytal he noted a 50 per cent decrease in the duration of sleep and the animals tolerated doses which were almost twice the LD_{50}.

A degree of tolerance was found by Stanton (12) in rats and by Masuda, Budde, and Dille (5) in rabbits following repeated injections of pentobarbital sodium (30% of LD_{50}). These results have been confirmed by Moir (14). However, Barlow (15) found the factor of tolerance to be negligible following repeated administrations of the drug to 6 rabbits.

Daily intravenous injections of nembutal were made for three months in 5 dogs by Ettinger (13). No difference in the duration of sleep was noted following injections of the drug as the experiments progressed. Vander Brook and Cartland (23) found that dogs uniformly became refractory to repeated injections of cyclopal but not to pentobarbital.

That guinea pigs develop a tolerance to nembutal was demonstrated by Carmichael and Posey (16). In all animals the period of hypnosis was markedly shorter on the second injection of 15 mgm. per kgm. of pentobarbital sodium than on the first injection. However, they found these animals not protected against a dose of 60 mgm. per kgm. (MFD.).

Daily administrations of ortal and evipal produced no significant changes in the sleeping time when given to rabbits (5). Nor has tolerance or cumulative effects to evipal been demonstrated in mice (17).

Dogs apparently develop a tolerance to evipal quite readily as has been demonstrated by Dallemagne (18) and by Green and Koppanyi (1). A similar effect has also been demonstrated in rabbits to pernoston (5), in dogs to nostal, pentothal sodium (19) and to "numal" (18) and in guinea pigs to vinbarbital (delvinal) sodium (20).

Dallemagne (18) was unable to demonstrate cross tolerance between "evipan sodium" and "numal". Green and Koppanyi (19) observed evipal tolerant dogs to be tolerant also to pentothal sodium and to nostal; pentothal tolerant animals to evipal; and nostal tolerant animals were also found tolerant to evipal, to pentothal and to barbital.

Because of the differences in the method of experimentation employed in the various studies to determine whether or not experimental animals can acquire a tolerance to barbiturates and because conclusions were based on results from too few animals in most cases this work was undertaken in an effort to correlate and clarify conflicting data. Some experiments were also performed in which, after the animals showed a definite decrease in the period of sleep produced by a barbiturate, the LD_{50} of that drug was given. Additional experiments were performed to determine if an animal tolerant to one barbiturate was also tolerant to others.

The sodium salts of the following barbituric acid derivatives were investigated: 1) Sec-butyl ethyl (butisol); 2) Isoamyl ethyl (amytal); 3) cyclopentenylallyl (cyclopal); 4) ethyl (1-methyl butyl) (pentobarbital); 5) N-hexylethyl (ortal); 6) Propyl-methyl-carbinyl allyl (seconal); 7) N-methyl-cyclohexenyl-methyl (evipal).

Dogs, rabbits and albino rats were used in this investigation. Food was withheld 12 hours before each experiment except in those instances in which the drug was administered twice daily. The animals were weighed before each administration of a drug. To dogs and rabbits the drugs were given intravenously and to rats intraperitoneally. Only sodium sec-butyl-ethyl barbiturate and sodium ethyl (1-methyl butyl) barbiturate were studied in dogs. The solutions of these drugs were freshly prepared and injected slowly in 5 per cent solutions and the dose was usually 40 per cent of the LD_{50}.

The rabbits were divided into groups of 9 to 18 animals. Twenty-seven such groups were investigated. On these all of the above barbiturates were studied. Five per cent solutions of the drug were freshly prepared and slowly injected intravenously. The total amount injected was either 40 or 50 per cent of the LD_{50} for the particular barbiturate under investigation. In each instance the total dose was uniform per kilogram weight throughout each set of experiments. The injections are made every other day when investigating butisol, amytal, cyclopal and, in one group of animals, pentobarbital. Pentobarbital, seconal, ortal, and in one series of animals, evipal were injected once each day. By experimentation it was found that evipal gave best results when injected twice daily. When the effect of LD_{50} was studied on these tolerant animals the drug was injected at an absolutely uniform rate of 0.8 cc. (5% solution) per minute or 40 mgm. per minute.

All of the above barbiturates except isoamyl ethyl barbiturate were also studied in rats. The animals were weighed before each injection and a freshly prepared 1 per cent solution was used in all instances except with "ortal sodium" in which a 2 per cent solution was employed. In all experiments the total dose was 60 per cent of the LD_{50}.

A decrease in the duration of hypnosis was taken as an indication of tolerance. The criterion of the duration of action was much the same as that used by Fitch and Tatum (21). The animals were checked at the time they went to sleep and again as soon as they could raise their heads, sit upright, maintain that posture and hop or walk about when disturbed. The rats were counted awake as soon as they could right themselves and crawl around. Since the animals are usually still definitely depressed, the actual periods of the barbiturate depression are, of course, much longer than the times secured by these methods. All of the animals were watched continuously from the time of the injection until they were checked off as awake.

Dogs quickly acquire a tolerance to butisol sodium. This can be seen in table 1. In the 14 animals studied the duration of sleep following the third injection of butisol was 43 per cent shorter than that following the first injection. If the first ten animals in this table are considered the durations of sleep from the second, third and fourth injections were shorter than the first by 28, 35, 64 per cent respectively. That this acquired tolerance can be lost quickly is seen from the results obtained after the fifth injection of the drug in the first four dogs in the table. Within 19 days the acquired tolerance was almost if not entirely lost.

In our experiments it was possible to demonstrate acquired tolerance by rabbits to all of the barbiturates studied (table 2). If the first and subsequent injected doses were adequate and were properly spaced every group of animals and each animal in each group showed a decreased sleeping time upon repeated administrations of the drug. After the third injection the average duration of hypnosis was shortened by 36 per cent with amytal in 58 rabbits, by 47 per cent with butisol sodium, in 81 animals, by 47 per cent with pentobarbital sodium in 85 animals, by 45 per cent with seconal sodium in 70 animals, by 46 per cent with ortal sodium in 21 animals, by 37 per cent with cyclopal in 12 animals and by 36 per cent with evipal sodium in 19 animals.

Table 3 illustrates the effects of repeated intraperitoneal injections of the barbiturates in rats. In these the average decrease in the period of sleep with butisol sodium in 98 animals was 38 per cent, with pentobarbital sodium in 26 animals 57 per cent, with cyclopal in 18 animals 25 per cent, with ortal sodium in 12 animals 19 per cent, and with seconal sodium in 12 animals 15 per cent.

If a decrease in the sleeping time can be considered an indication of acquired tolerance to a barbiturate our results then show conclusively that such tolerance can be developed in experimental animals. However, as the question of whether or not an animal can also acquire any degree of tolerance to toxic doses of a barbiturate was still to be determined we undertook to investigate this problem in rabbits (table 4). In all experiments when the LD_{50} was injected intravenously it was done at a uniform rate (0.8 cc per minute) and the concentration of the drug was 5 per cent.

Sixteen animals averaging 2.2 kgm. were given intravenously 40 mgm. per kgm. of amytal sodium every other day. The first injection was followed by sleep of 150 minutes duration whereas the fourth injection produced sleep for only 97 minutes. Two days later these animals were given 80 mgm. per kgm. (LD$_{50}$) of amytal sodium. It was rather surprising to find that this dose killed 80 per cent of these animals. We then proceeded to determine the LD$_{50}$ for our own particular strain and size of animal. Using 20 animals in each group we found 70 mgm. per kgm. killed 16.5 per cent, 75 mgm. per kgm., 50 per cent and again 80 mgm. per kgm. killed 80 per cent of the animals. Thirteen animals averaging 1.9 kgm. were given 37.5 mgm. per kgm. (50% of LD$_{50}$) of amytal sodium every other day. Following the first injection the animals slept 102 minutes and after the third injection 80 minutes. Two days later these animals were given intravenously 75 mgm. per kgm. (LD$_{50}$) of amytal sodium. Seven of the 13 animals died, a mortality of 54 per cent.

TABLE 1

The effects of repeated administrations of Butisol sodium on the duration of hypnosis in dogs

Unless otherwise indicated the dose injected intravenously was 36 mgm. per kgm. (40% of LD$_{50}$). The time interval between injections was 48 hours. In some animals pentobarbital sodium was injected intravenously in doses of 20 mgm. per kgm. (40% of LD$_{50}$) the day following the last injection of Butisol sodium. The duration of hypnosis for each such experiment is given in column 6. A similar dose has been injected one month before the beginning of Butisol administration. The duration of hypnosis then produced is given in column 7.

SEX	WT.	DURATION OF HYPNOSIS IN MINUTES						
		1	2	3	4	5	6	7
	kgm.							
♂	10	502	342	159	169	414†		
♀	13*	550	364	259	150	333†		
♂	10	600	480	480	341	720†		
♀	9	447	307	257	145	347†		
♀	10	408	358	340	130	135	20	72
♀	14.5	445	306	300	185	230	87	102
♀	10	400	290	332	139	95	51	100
♀	8.5	260	208	175	113	113	67	60
♀	10	308	176	180	120			
♀	8	564	393	296	109			
♂	17	210	205	130			53	70
♂	8	540	‡	240				
♂	8	523	‡	265				
♀	9	370	‡	128				

* 60% LD$_{50}$ (54 mgm./kgm.).

† Nineteen days between injections.

‡ *Injection made but duration of action not determined.*

Four groups of rabbits were used in studying resistance to toxic doses of butisol sodium. As can be seen in table 4 there was no added resistance to such doses since LD$_{50}$ killed approximately 50 per cent of the 45 animals studied.

Since 45 mgm. per kgm. (LD$_{50}$) of pentobarbital sodium killed only 20 per cent of the 16 tolerant animals in our first group, the LD_{50} for this drug was further investigated. Sixteen animals were used in each group and we found 45

TABLE 2

The effects of repeated administrations of a barbiturate on the sleeping time in rabbits

The animals were of the same strain and each lot was of fairly uniform weight (varying ±0.3 kgm. from that of the average). The injections were made intravenously every other day with amytal sodium, butisol sodium and cyclopal, daily with seconal sodium and pentobarbital sodium and twice daily with evipal sodium.

DRUG	NUMBER OF ANIMALS	AVERAGE WT.	DOSE	DURATION OF HYPNOSIS IN MINUTES FOLLOWING INJECTION					DECREASE IN SLEEPING TIME
				1	2	3	4	5	
		kgm.	*mgm./kgm.*						*per cent*
Amytal sodium	12	2.4	40	158	93	88	82		49
	16	2.2	40	150	101	98	87		42
	17	2.5	37.5	121	79	82			30
	13	1.9	37.5	112	86	80			28
Butisol sodium	10	2.2	36	113	62	51			55
	14	2.0	36	71	57	49	40		43
	12	3.1	36	143	85	77	76	72	50
	11	3.3	45	185	125	120	101		46
	12	1.9	36	96	72	57	57		41
	12	3.0	36	144	96	63	60		60
	10	3.4	45	261	171	170	147		43
Pentobarbital sodium	12	3.0	18	84	52	32	32		62
	11	3.1	18	59	34				32
	16	2.3	22.5	91	60	54	53		42
	14	2.0	26	106	62	56	57		46
	17	2.6	22.5	85	58	51			40
	15	1.9	25	107	70	53	53		50
Seconal sodium	12	2.7	18	34	28	28	26		23
	11	2.3	22.5	100	55	44	44	46	54
	18	2.3	22.5	95	52	47	50		47
	9	2.3	22.5	68	49	47	46		32
	10	2.2	22.5	121	68	62	61		50
	10	2.2	22.5	97	54	47	50		50
Ortal sodium	9	2.7	45.0	71	50	32	37		48
	12	3.1	45.0	65	44	35	36		45
Cyclopal	12	2.7	40	116	75	73			37
Evipal sodium	10	2.0	40	39	38	31	25	25	
					39	28	25	26	36

mgm. per kgm. killed 25 per cent, 48 mgm. per kgm. 33 per cent, 50 mgm. per kgm. 80 per cent of the animals in each group. Using these newly determined

LD_{50}'s for our animals we found 52 mgm. per kgm. killed 86 per cent of our supposedly tolerant animals and 50 mgm. per kgm. killed 80 per cent.

Animals showing a decreased sleeping time following repeated administrations of seconal sodium show no appreciable change in their resistance to toxic doses of the drug (table 4). In the 47 rabbits so investigated 45 mgm. per kgm. (LD_{50}) of seconal sodium injected at a uniform rate killed between 33 and 70 per cent of the animals. As a control we injected intravenously 45 mgm. per kgm. (LD_{50}) of seconal sodium into 12 normal untreated rabbits. Only 42 per cent of these animals died as the result of the injection. If we compare the sleeping time of the treated animals which survive the injection of the LD_{50} (table 4, last column) with that of untreated ones a marked difference is seen. In our experiments the duration of sleep in the control animals which survived the LD_{50} of amytal sodium, butisol sodium, pentobarbital sodium and seconal sodium was 253, 432, 184, and 187 minutes respectively. A comparison of the duration of sleep in these animals with those tabulated in the last column in table 4 shows that in all instances the treated animals slept a much shorter time following even toxic doses.

TABLE 3

The effects of repeated intraperitoneal administrations of barbituric acid derivatives on the sleeping times of albino rats

Each animal was weighed before each injection. All of the drugs used were injected every other day unless otherwise indicated

BARBITURATE	NUMBER OF ANIMALS	AVERAGE WEIGHT	DOSE	DURATION OF HYPNOSIS IN MINUTES FOLLOWING INJECTION				
				1	2	3	4	5
		gms.	*mgm./kgm.*					
Butisol sodium	12	118	40	250	108	105	250*	
	13	220	33	140	31†			
	18	167	40	156		126‡	234*	
	11	190	40	234	152	132‡	124	201*
	13	210	40	213	143	128	159‡	124
	17	223	40	201	156	140	98	129
	14	190	40	256	190	135	125	
Pentobarbital sodium	14	194	29	145	86	97‡	95	73
	12	230	29	104	59	58	48	34
Cyclopal	18	220	75	199	162	151		
Ortal sodium	12	277	150	104	105	86§	85§	
Seconal sodium	12	150	66	310	251	264		

* 30 day interval since last injection.

† 9 animals failed to show hypnosis.

‡ 3 day interval between injections.

§ Two animals failed to show any hypnosis.

From these results it appears that the mechanism on which the barbiturate act in causing death acquires no resistance to the drug upon repeated injections. On the other hand, the mechanism upon which the drug acts in causing sleep becomes resistant by repeated administrations of the compound. These results are similar to those found by Swanson, Weaver, and Chen (2) with amytal in dogs, but contradictory to those reported by Fitch (4) with amytal, neonal and noctal in rabbits. The lack of agreement is probably due to the fact that the latter investigator used but 2 to 4 animals in each group and that the drugs were administered orally. It is a well known fact that the stomachs of rabbits are never completely empty and the rate of absorption of the drug will be influenced by the amount of material in the stomach which in turn affects the mortality rate.

TABLE 4

The influence of repeated injections of barbiturates upon the mortality per cent of LD_{50}

Rabbits were used and the intravenous injections were at a uniform rate, 0.8 cc per minute. Five per cent solutions were employed in each case

BARBITURATE	NUMBER OF ANIMALS	AVERAGE WT.	DOSE	DURATION OF HYPNOSIS INJECTION				LD_{50}	MORTALITY	SLEEPING TIME OF SURVIVALS
				1	3	4	5			
		kgm.	*mgm./kgm.*					*mgm./kgm.*	*per cent*	
Amytal sodium	16	2.2	40	150		97		80	80	150
	13	1.9	37.5	112	80			75	54	
Butisol sodium	12	3.1	36	143			72	90	40	330
	11	3.3	45	185		101		90	46	294
	12	1.9	36	96		57		90	50	290
	10	3.4	45	261		147		90	50	300
Pentobarbital sodium	16	2.3	22.5	91		53		54	20	106
	14	2.0	26	106		57		52	86	124
	15	1.9	25	107		53		50	80	106
Seconal sodium	18	2.3	22.5	95		50		45	47	124
	9	2.3	22.5	68		46		45	33	101
	10	2.2	22.5	121		61		45	70	121
	10	2.2	22.5	97		50		45	70	107

Cross tolerance. Since discrepancies exist in the literature (18, 19, 22) as to whether or not cross tolerance to barbiturates can be produced in experimental animals further experiments were performed in the hope of obtaining conclusive results. Five dogs were given, intravenously, 20 mgm. per kgm. of pentobarbital sodium and the duration of the induced sleep recorded. One month later after a series of injections had made these animals tolerant to butisol sodium the same dose of pentobarbital was administered as had been originally (table 1).

The average sleeping time before the butisol treatments was 81 minutes and after it was only 55 minutes.

A few experiments were also performed on rats. All of the animals which showed a decrease in the duration of sleep following repeated injections of butisol sodium showed a decrease following pentobarbital sodium and vice versa.

Twelve rats whose average sleeping time decreased from 213 to 124 minutes from the first to the fifth injection of butisol sodium were given 29 mgm. per kgm. of pentobarbital sodium. As a result two of the animals failed to show any hypnosis and the remaining animals slept an average of 34 minutes. These injections were repeated at 48 hour intervals and the sleeping time gradually increased until it reached 65 minutes on the fourth injection. The animals were then rested for one month after which they were again injected with 29 mgm. per kilogram of pentobarbital sodium. As a result of this injection one animal died and the remaining animals slept for an average of 104 minutes. In another group of 14, each animal was given 29 mgm. per kgm. of pentobarbital sodium every other day for five injections and during this period the sleeping time decreased from 145 minutes to 73 minutes. Butisol sodium, 40 mgm. per kgm., was then substituted for the pentobarbital and the average sleep was 190 minutes. After the sixth injection of butisol the time had been reduced to 135 minutes. For three weeks the animals were kept undosed and then the same dose of butisol was administered resulting in a sleep with an average duration of 256 minutes. From these results in rats it can be seen that tolerance acquired for the one barbiturate also provided tolerance for the other.

TABLE 5

The effect of repeated administrations of one barbiturate on the duration of the sleeping time of another

Rabbits of the same strain and weight (±0.3 kgm. of the average) were used in each group

BARBITURATE	DOSE	NUMBER OF ANIMALS	AVERAGE WT.	DURATION OF HYPNOSIS		24 HOURS AFTER 3RD		DURATION OF SLEEP	DURATION OF SLEEP	
				1st Injection	3rd Injection		Dose		14 days later	21 days later
	mgm./kgm.		*kgm.*	*minutes*	*minutes*		*mgm./kgm*	*minutes*	*minutes*	*minutes*
Butisol sodium	36	10	2.2	113	51	Pentobarbital sodium	18	21	40	
	36	12	2.2	144	60		18	28	50	50
	36	14	2.0	120	40		18	21	54	54
	36	12	3.1	143	72		18	32	84	
Pentobarbital sodium	18	12	3.0	84	32	Butisol sodium	36	84		153
	22.5	17	2.6	85	51	Amytal sodium	37.5	69		127
Amytal sodium	37.5	17	2.5	121	82	Pentobarbital sodium	18	43	85	

Most of our work on cross tolerance to barbiturates was done on rabbits. It will be seen in table 5 that the 48 animals made tolerant to butisol sodium upon repeated intravenous injections of the drug were also tolerant to pentobarbital sodium when it was similarly administered. The average sleeping time following the intravenous administration of the latter drug immediately after the animals were made tolerant to the former was 26 minutes, but fourteen days later, during which period no barbiturate was administered, the same dose of the drug produced an average sleeping time of 58 minutes. From table 5 it will be seen that animals tolerant to pentobarbital were also tolerant to butisol and amytal sodium and likewise animals made tolerant to amytal sodium were also tolerant to pentobarbital sodium.

CONCLUSIONS

1. If a reduction in sleeping time can be taken as a criterion of acquired tolerance to barbiturates in experimental animals then we have demonstrated (a) that dogs can acquire tolerance to butisol sodium and pentobarbital sodium (b) rats can acquire a tolerance to butisol sodium, pentobarbital sodium, cyclopal, ortal sodium and seconal sodium and (c) rabbits can acquire a tolerance to amytal sodium, butisol sodium, pentobarbital sodium, seconal sodium, cyclopal and evipal sodium.
2. A tolerance to the barbiturate, as judged by the shortened sleeping time, is no protection against the LD_{50}.
3. A dog, rabbit or rat made tolerant to one barbiturate will very likely show some tolerance to all other barbiturates. Cross tolerance was shown in rabbits for butisol sodium, pentobarbital sodium and amytal sodium, in dogs and rats for butisol sodium and pentobarbital sodium.
4. In developing tolerance in rabbits the time interval can be longer between administrations when long acting barbiturates are used than when short acting barbiturates are studied. To develop tolerance in rabbits to evipal sodium the drug must be administered twice each day.
5. Tolerance to the barbiturate is rapidly lost in experimental animals following cessation of administration.

We wish to thank the following for the barbiturates used in these experiments: McNeil Laboratories, Butisol Sodium; Eli Lilly and Co., Sodium Amytal, Seconal Sodium and Pentobarbital Sodium; The Upjohn Co., Cyclopal; Parke Davis and Co., Ortal Sodium; and Winthrop Chemical Co., Evipal.

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PRELIMINARY STUDIES OF THE ANESTHETIC ACTIVITY OF FLUORINATED HYDROCARBONS[1]

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Numerous reports relative to the anesthetic activity of chlorine, bromine and iodine derivatives of the lower members of the methane and ethylene series of hydrocarbons have been made, the most recent being that of Abreu et al. in 1944 (1). Fluorine derivatives of the lower hydrocarbons have however received no such extensive study. Most of the reports on fluorinated hydrocarbons deal with those members used as refrigerants (Brenner (2), Booth and Bixby (3)). Struck and Plattner (4) investigated C_4F_{10}, C_5F_{10} and C_6F_{12} for their anesthetic activity. These compounds did not produce full anesthesia, and the mice had convulsions upon stimulation. Henne and his associates (5) have included remarks relative to the physiological activity, or lack of such activity, of certain fluorinated hydrocarbons in their reports on the preparation and chemical characteristics of these substances.

We have had the opportunity during the past two years of testing some forty-six fluorine compounds for their anesthetic activity. These substances were prepared by Dr. E. T. McBee and his associates at Purdue University, who very kindly permitted us to test them for their anesthetic activity. Over one-half of these agents are new and data on their chemical characteristics as well as the methods used in their preparation will be published by Dr. McBee and his associates.

Although most of these substances contain other halogens in addition to fluorine, there have been sufficient fluorine compounds in this series to show that these are not as inert physiologically as previously thought.

PART I

METHODS. *Animals.* Adult male white mice were used throughout this study.

Determination of anesthetic and fatal concentration: The procedure was to determine the concentrations of the compound necessary to anesthetize 50% of the mice upon ten minutes' exposure and that necessary to cause death in 50% in ten minutes. Two mice were placed into a closed 2.7 liter bottle, 12 cm. internal diameter, and the compound injected through a rubber dam and volatilized (except for the four low boiling ones). The bottle containing the mice was then attached to a revolving machine, 14 revolutions per minute, and the time noted at which the mice were unable to maintain an upright position and rolled over continuously for 15 seconds. After determining the concentration necessary for anesthesia, that necessary for respiratory arrest of 50% of the mice in 10 minutes' exposure was found. The mice which survived the FD50 concentration for 10 minutes were

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[1] Funds for carrying out this work were kindly supplied by the Mallinckrodt Chemical Works.

TABLE 1

The concentrations of various halogenated hydrocarbons necessary to produce anesthesia and respiratory arrest in 50 per cent of the mice exposed

Also the time necessary for induction of anesthesia as well as times necessary for recovery of pain sensation and ability to walk after exposure to the FD50 for 10 minutes are given.

FORMULA	BP°	AD50	FD50	FD50/AD50	NO MICE	TIME† A.	P.	W.	REMARKS*
Fluorine substituted hydrocarbons and ethers									
		vol. %	*vol. %*						
CF_3CH_3	−46	50–60			5				conv. on recovery
$CF_3CH{=}CH_2$	−24	60			13				conv. on recovery
$CF_3CH_2CH_3$	−12	50			5				
$CF_3CH_2CF_3$	−.5	11	44	4	14	30″	30″	90″	
$CH_3CF_2CF_2CH_3$	17	20	60	3	18	30″	10″	90″	
$CH_3CF_2CH{=}CH_2$	24	8.3	25	3	26	20″	20″	2′–3′	
$CH_3CF_2CH_2CH_3$	30.8	6	12	2	24	1′	20″	60″	
$CF_3CH_2CF_2CH_3$	40	5			9		30″	60″	
$CF_3CH_2OCH_3$	30	8	16	2	20	20″	2′	4′	
$CF_3CH_2OC_2H_5$	50.3	4	8	2	16	30″	20″	60″	
$CHF_2CH_2OC_2H_5$	65	4	9	2.2	24	1′	2′	5′–20′	
Bromine substituted fluoro hydrocarbons									
CF_3CH_2Br	26.5	2.8	11.7	4.2	74	15″	75″	3′	
$CF_3CHBrCH_3$	48.6	1.7	7.6	4.5	22	15″	60″	2′–3′	
CHF_2CH_2Br	57	1.3	4.6	3.5	90	45″	2′	8′	
$CF_3CH_2CH_2Br$	62	1.5	4.5	3	24	45″	45″	90″	D. D.
CF_2ClCH_2Br	68	.8	3.7	4.6	72	20″	5′	10′	
CF_3CHBr_2	73	.4	2	5	44	20″	4′	10′	
$CH_3CF_2CH_2Br$	75.4	1.25	5.8	4 6	36	20″	3′	10′	
$CF_2ClCHBrCH_3$	89	.56	2.2	4	26	15″	7′	10′–20′	
$CF_3CHBrCH_2Br$	116	.1	.67	6.7	52	45″	10′	20′	D. D.
Chlorine and iodine substituted fluoroethane									
CF_2ClCH_3	−9.6	25			12				conv. on recovery
CF_3CH_2Cl	6 1	8	25	3	23				conv. on recovery
CF_3CHCl_2	28 7	2 7	7.7	2 8	24	30″	70″	90″	
$CFCl_2CH_3$	31.7	2.5	5	2	26	75″	75″	90″	
CHF_2CH_2Cl	36	2.15	7.5	3.5	56	45″	3′	5′	
CF_2ClCH_2Cl	46.8	1.3	4.3		8	60″	4′	7′	D. D.
CF_3CH_2I	55	1.25	5	4	30	15″	2′	5′	

* conv. on recovery = convulsions during the recovery period.

D. D. = delayed death, within 24–48 hours after anesthesia

† A = Time necessary for induction of anesthesia of mice when exposed to LD50 concentration. P = Time necessary for recovery of pain sensation after exposure to the LD50 concentration for 10 minutes W = Time necessary for the return of normal walking after exposure to the LD50 concentration for 10 minutes.

TABLE 1—*Continued*

FORMULA	BP*	AD50	FD50	FD50/AD50	NO. MICE	TIME†			REMARKS*
						A.	P.	W.	
Chlorine substituted fluoropropane									
		vol.%	vol.%						
$CF_2ClCH_2CH_3$ ..	25.–26.	8	16	2	10	30″	30″	90″	
$CF_3CH_2CH_2Cl$. .	45	3	9.7	3.2	20	20″	30″	60″	D. D.
$CF_3CCl_2CH_3$ . .	48.1	4	10	2.5	32	20″	20″	60″	
$CHF_2CHClCH_3$.	52	1.7	7.6	4.5	30	15″	3′	7′	
$CH_3CF_2CH_2Cl$.	55	2.15	8.4	4	50	20″	45″	4′	
$CFCl_2CH_2CH_3$ .. .	66.7	1.5	5.3	3.5	30	20″	90″	2′–3′	
$CF_2ClCHClCH_3$ . . .	69.5	.96	4.1	4.3	66	20″	3′	6′	
$CF_3CH_2CHCl_2$ .. .	71	.56	2.4	4.3	36	45″	90″	5′	D. D.
$CF_3CHClCH_2Cl$.	75.5	.4–.5	2.2	4.5	20	30″	3′	7′	
$CF_2ClCH_2CH_2Cl$	80.8	.9	2.25	2.5	30	45″	45″	3′	D. D.
$CF_2ClCHClCFCl_2$.	85	.6	1.8	3	30	90″	2′	6′	D. D.
Chlorine substituted fluorobutane									
C_4HClF_6 . .	34								convulsions
$CH_3CF_2CFClCH_3$	53	3.4	11.8	3.5	26	30″	90″	4′	
$CH_3CFClCH_2CH_3$	67	1.8	6	3.3	44	30″	1′	5′	
$CH_3CF_2CHClCH_3$	71.4	.74	3.7	5	34	30″	90″	3′	
$C_4H_7F_2Cl$	89	1	4	4	28	45″	2′	6′	
$CH_3CF_2CH_2CHCl_2$	117	.35	1.3	3.7	32	90″	5′	10′	D. D.
$CH_3CF_2CHClCH_2Cl$	120	.2	1	5	40	60″	5′	15′	
$C_4H_5F_2Cl_3$.	137	12	.7	5.8	44	2′	8′	15′	
Diethyl ether and chloroform for comparison									
$C_2H_5OC_2H_5$	35	3.2	11.2	3.5	38	45″	3′	5′	
$CHCl_3$	61 2	.78	2.6	3 3	26	45″	2′–3′	5′	

removed from the bottle and examined to determine the time necessary to recover pain sensation (pressure on tail) and ability to walk.

With the lower boiling compounds the anesthetic mixture was prepared by letting the compounds volatilize into a deflated rubber bag and then transferring known volumes of the gas to a 500 cc. jar containing the mice in an O_2 atmosphere.

The number of mice used with the different agents varied from 5 to 90, this variation depending upon the quantity of material available.

The results of this study are presented in table 1, including the chemical formula, boiling point, Anesthetic Dose 50, and Fatal Dose 50, their ratio, induction time when exposed to the FD50 concentration, recovery time for pain sensation, and ability to walk after 10 minutes' exposure to FD50 concentration, and finally remarks regarding convulsions and delayed deaths.

In the last two lines in table 1 data obtained with diethyl ether and chloroform are included for comparison.

Upon examining the data in table 1 certain points may be made:

1. All the compounds, except C_4HClF_6, produced anesthesia in the mice.

2. The low boiling compounds produce convulsive movements particularly during the period of recovery from the agents; convulsive movements were noted by Struck and Plattner (4) who used C_4F_{10}, C_5F_{10} and C_6F_{12}, and Brenner (2) investigated the mechanism by which convulsions were produced by CF_2Cl_2.

3. There is an increase in potency of the members of each group as the boiling point increases.

TABLE 2

The effect of varying the type and number of secondary halogen substituents upon the concentration of the agents necessary for anesthesia and respiratory arrest

FORMULA	BOILING POINT	AD50	FD50	FD50/AD50
Trifluoroethanes				
		%	%	
CF_3CH_3	−46°	50		
CF_3CH_2Cl	6.1	8	25	3
CF_3CH_2Br	26.5	2.8	11 7	4 2
CF_3CH_2I	55	1.25	5	4
CF_3CHCl_2	28	2.7	7 7	2 8
CF_3CHBr_2	73	.4	2	5
Trifluoropropanes				
$CF_3CH_2CH_3$	−12	50		
$CF_3CH_2CH_2Cl$	45	3	9.7	3.2
$CF_3CH_2CH_2Br$	62	1 5	4.5	3
$CF_3CHClCH_2Cl$	75	.4- 5	2.2	4.5
$CF_3CHBrCH_2Br$	116	.1	67	6 7
Difluoropropanes				
$CH_3CF_2CH_2Cl$	55	2 15	8.6	4
$CH_3CF_2CH_2Br$	75	1.25	5 8	4.6
$CF_2ClCHClCH_3$	69 5	96	4.1	4.3
$CF_2ClCHBrCH_3$	89	.56	2 2	4

4. Approximately one-half of the substances examined have as great margins of safety, FD50/AD50 index, as do ether and chloroform when tested by this procedure.

5. Only the bromine substituted fluorohydrocarbons as a group have margins of safety greater than ether or chloroform.

6. The time necessary for recovery of pain sensation and the ability to walk is much greater following 10 minutes' exposure to the higher boiling substances than following a like period of exposure to the lower boiling substances even though the rates of induction with all compounds are approximately the same.

Data for fifteen compounds from table 1 are presented in table 2 where the compounds are arranged in groups of two or more in which the members of each group differ only in the type of secondary halogen substitution. From the data in table 2 one may state that:

1. The introduction of a second halogen atom, either chlorine, bromine or iodine, into a fluorohydrocarbon increases the potency very markedly.
2. Bromine substitution increases the potency 2–4 times more than chlorine in each of the six pairs of compounds studied.
3. The dibrom and dichlor substituted fluorohydrocarbons are more potent than their monosubstituted homologues.

PART II

We have selected several compounds which had high FD50/AD50 ratios for study on dogs for their anesthetic effect.

Methods. Because only limited quantities of the substances were available small dogs were selected for use.

Preparations were made for recording blood pressure, respiration, and for observing electrocardiac changes (Lead II) by use of a Sanborn Cardioscope. As a rule, the trachea was cannulated using procaine-HCl as an anesthetic. After preliminary control observations of blood pressure, respiration, heart rate and electrocardiographic pattern were obtained, attempts to produce anesthesia were begun. A to-and-fro closed system was used and after connecting the gas bag, filled with oxygen, and soda lime cannister to the tracheal cannula the drug was added to the system at the distal end of the cannister, at the rate of .05—.1 cc. per kilo body weight per 1 to 2 minutes until anesthesia (Stage III$_2$) was induced; at this time further records of blood pressure, respiration, heart rate and electrocardiographic observations were made. Then further quantities of the drug were added until Stage IV (respiratory arrest) was approached or reached. Records were made of Stage III$_2$, III$_3$, III$_4$, and IV.

The results of these studies on dogs are shown in table 3, from which the following conclusions are drawn:

1. All of the eighteen compounds produced anesthesia in dogs.
2. These agents produced a fall in blood pressure which became more marked in the deeper levels of anesthesia.
3. Abnormalities of the cardiac rhythm as shown by electrocardioscopic examination were very frequently produced, and ventricular fibrillation was observed in four instances.
4. Dogs under nine of the eighteen compounds showed hyper-activity of the skeletal muscles, and this was more marked during the deeper levels of anesthesia.
5. The results obtained with four of these compounds, $CF_3CHBrCH_3$, CF_3CHBr_2, $CF_3CHClCH_2Cl$ and $CHF_2CHClCH_3$, are such that we feel further investigations of them as possible anesthetic agents are indicated.

SUMMARY AND CONCLUSIONS

1. The anesthetic activity of forty-six hydrocarbons containing fluorine alone, or in addition to other halogens, has been determined in mice.
2. Eighteen of these forty-six compounds have been used on dogs to study

TABLE 3

Effect of various halogenated hydrocarbons upon the blood pressure and rhythm of the heart during anesthesia by these agents

COMPOUND	EXPT. NO.	BLOOD PRESSURE					CARDIAC ARRHYTHMIA†	REMARKS
		Control	III_2	III_3	III_4	IV*		
CF_3CH_2Br	1	125	100	80	70		None	Skeletal movement III_4
	2	140		125	70		V. ex.; v. tach.	Tremor stages III_3 & IV
	3	120		80		55	V. ex.	Tremor stages III_3 & IV
	4	130		110	70	40	V. ex.; nodal	Rigid stage IV
CHF_2CH_2Br	1	130		120	100		V. ex.; nodal	Rigid; convulsion
CF_3CH_2I	1	130		105		70	Nodal; v. ex.	
	2	125		100	75	65	V. ex.	
CF_2ClCH_2Br	1	120	115	100	85		A.V. dis.; nodal	
	2	100	90	90	60–40		4:1 block; nodal v. fib.	
	3	120		90	60		V. ex.; v. tach.; nodal	
	4	120	100	90	60–40	30	Nodal; v. tach.	
$CFCl_2CH_3$	1	150	90		55		Nodal	Rigid
$CH_2ClCF_2CH_3$	1	115		85	70		None	
	2	100	90	80	60		Nodal	
	3	130	50		60		A. V. block	
	4	110	100	30	20		V. tach.; nodal	
$CClF_2CHClCH_3$	1	140	90	75	40		Low voltage Stage III_{2-4}	
	2	90	70	60	55		None	
	3	120		70	40		None	
	4	100	70	70			None	
$CF_3CH_2CHCl_2$	1	130	120	110			None	
	2	120					V. fib. stage II	
	3	120					Nodal; v. ex.; v. fib. stage II	
	4		100	90	80		Low voltage	
	5				70		None	

* Classification after Seevers, et al. (6).

† V. ex. = ventricular extra systole; v. tach. = ventricular tachycardia; nodal = auriculoventricular nodal rhythm; A.V. dis. = auriculoventricular dissociation; A.V. block = auriculoventricular block.

TABLE 3—*Continued*

COMPOUND	EXPT. NO.	BLOOD PRESSURE					CARDIAC ARRHYTHMIA†	REMARKS
		Control	III_2	III_3	III_4	IV*		
$CF_2ClCH{=}CH_2$	1	130	90	85			Nodal; v. ex.	Tremor
$CF_3CH_2OC_2H_5$	1	150	150	140			None	Rigid with convulsions on stimulation
$CH_3CF_2CFClCH_3$	1	140	105	50			Nodal	Tremor: rigid
	2						V. tach.; v. fib.	V. fib. during induction
	3	135	50	40			Nodal	
$CH_3CF_2CF_2CH_3$	1	130	60			50	Nodal; v. ex.; A.V. dis.	Tremor; convulsion
$CF_3CHBrCH_3$	1	140		125	80	45	None	Rapid rise in Bp. when anesthesia stopped
	2	110	95	85	75	45	None	Rapid rise in Bp. when anesthesia stopped
	3	140		90	70–80	20	None	Rapid rise in Bp. when anesthesia stopped
	4	130		90	80	40	None	Rapid rise in Bp. when anesthesia stopped
	5	120	100	90	80	50	None	Rapid rise in Bp. when anesthesia stopped
CF_3CHBr_2	1	100	85	80	100	75	None	
	2	120	100	100		65	None	
$CF_3CHClCH_2Cl$	1	115		100	85–100	60	None	
	2	115	90	105	70	20	None	
$CHF_2CHClCH_3$	1	125		120	95	40	None	
	2	115	100	100	85	70	None	
$CF_2ClCHBrCH_3$	1	130		75	60	40	Low voltage stage III_4	Tremor on recovery
	2	160		105	75	70	M.F. v. ex. induction	Tremor on recovery
	3	130		65	75		A.V. nodal; irregular	
$CH_2BrCF_2CH_3$	1	95		65	25		S.A. inhibition stage I	
	2	150		100	60		V. ex., stage I; low voltage	Leg movement, stage III_4

their effect upon the blood pressure and changes in the cardiac rhythm as show by electrocardioscopic examination.

3. Data obtained with four of these compounds are such that further stud of them as anesthetic agents is indicated.

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ERRATUM

In the article by Gladys A. Emerson and Dorothy G. Smith, "Induction of Nutritional Deficiency by Oral Administration of Streptomycin", which appeared in The Journal of Pharmacology and Experimental Therapeutics, 85: 336–342, 1945, on page 337, line 5, there should be included in the H4 diet—10 mg. Ca pantothenate and 10 mg. of nicotinamide per 100 gm. of ration.

CERTAIN PERIPHERAL AND CENTRAL NERVOUS SYSTEM EFFECTS OF β-DIETHYLAMINOETHYL PHENYL-α-THIENYLGLYCOLATE HCl AND PHARMACOLOGICALLY RELATED COMPOUNDS[1]

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Substitution of thiophene for the benzene nucleus has been shown by a number of investigations (1–5) to result in pharmacologic equivalence. This occurs even though such alteration has been made in series widely unrelated pharmacologically. Blicke and co-workers have prepared a series of basic esters of substituted α-thienylacetic, α-thienylglycolic (α-thienylhydroxyacetic) (6) and p-xenylacetic acids (7), which were tested for antispasmodic activity and toxicity by Lewis, Lands and Geiter (8) and Lands and Nash (9). Since the latter two groups of investigators indicated that some of their agents possessed potent antispasmodic properties, it was decided to extend their investigations to different smooth muscle preparations and species of animals. Three of their more active compounds selected include: β-diethylaminoethyl phenyl-α-thienylglycolate HCl, β-diethylaminoethyl phenyl-α-thienylacetate HCl and β-piperidinoethyl-α-methyl p-xenylacetate HCl. In addition, the known spasmolytics, atropine sulfate, β-diethylaminoethyl diphenylacetate HCl (trasentin) and β-diethylaminoethyl fluorene-9-carboxylate HCl (pavatrine) have been included in some experiments. Since experiments in rabbits indicated that β-diethylaminoethyl phenyl-α-thienylglycolate HCl was highly potent, most of the detailed studies in the dog and monkey were made with only this compound compared with one or more known spasmolytics.

METHOD. *Spasmolytic action* was determined in: 12 rabbits anesthetized with paraldehyde, 2 cc./kgm., intragastrically; 6 dogs pretreated with morphine sulfate, 4 mgm./kgm., subcutaneously; and 7 monkeys pretreated with morphine sulfate, from 4 to 10 mgm./kgm., 5 of which were sedated with sodium pentobarbital, from 10 to 20 mgm./kgm., intraperitoneally. No appreciable differences in spasmolytic response were noted between unanesthetized and sedated monkeys.

The activity of both the distal ileum and proximal portion of the ascending colon was recorded in the rabbit after abdominal incision and insertion of single small balloons into each of these structures. Recording was accomplished by connecting the catheters to Harvard membrane manometers. Air transmission was used throughout. In dogs and monkeys, balloons in tandem filled but not distended with air were connected to the same type manometer system used in the rabbit. In these two species, the balloons were introduced 20-30 cm. into the distal colon by way of the rectum.

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[1] Aided by a research grant from Frederick Sterns & Company, Division, Sterling Drug, Inc., Detroit.

[2] Research Fellow in Pharmacology.

Hyperactivity of the rabbit intestine was induced by pilocarpine HCl, 1 mgm./kgm. given intravenously. Morphine sulfate, in the dosage indicated above, was used as the spasmogenic agent in dogs and monkeys since Atkinson, Adler and Ivy (10) have indicated that hypertonus produced by this agent may be similar to the hypertonus and spasm which occurs in human colonic disease.

Mean blood pressure was recorded from the carotid or femoral artery by means of a mercury manometer in 4 rabbits anesthetized with paraldehyde, 2 cc./kgm., intragastrically, and in 3 dogs anesthetized with morphine sulfate, 4 mgm./kgm., subcutaneously, and sodium pentobarbital, 20 mgm./kgm., intravenously. Changes in cardiac rate were determined in 6 morphine treated dogs and 3 untreated dogs.

In order to obtain some indication of action on the central nervous system, unanesthetized monkeys or dogs were observed after the subcutaneous administration of β-diethylaminoethyl phenyl-α-thienglycolate HCl. An estimate of qualitative action in the central nervous system was obtained by determining: 1) whether the animal showed abnormal resentment to restriction by tying on a short tether or by being bound to an animal board; 2) whether he responded when caged to prodding by a small bar; 3) whether he was able to take proffered articles of food in the usual manner, and 4) whether the animal's unrestricted gait on the laboratory floor was influenced.

One monkey received daily amounts of β-diethylaminoethyl phenyl-α-thienylglycolate HCl which began at 1 mgm./kgm., intramuscularly, and were increased geometrically until death resulted.

The dosage of the various spasmolytics used varied from 0.1 to 1 mgm./kgm. and administration was either by the intravenous or subcutaneous route.

Results and discussion. *Rabbits.* The extremes of duration of colonic and ileal spasmolysis for this group of animals are shown in table 1. The *mean blood pressure* was uniformly elevated after the administration of the various spasmolytics. Elevations were as follows: From 4 to 30 mm. Hg above controls averaging 74 mm. Hg by β-diethylaminoethyl phenyl-α-thienylglycolate HCl, 1 mgm./kgm.; from 2 to 30 mm. Hg above controls averaging 66 mm. Hg by β-diethylaminoethyl phenyl-α-thienylacetate HCl, 1 mgm./kgm.; From 2 to 31 mm. Hg. above controls averaging 73 mm. Hg by β-piperidinoethyl-α-methyl p-xenylacetate HCl, 1 mgm./kgm.; from 8 to 14 mm. Hg above controls averaging 77 mm. Hg by β-diethylaminoethyl diphenylacetate HCl (trasentin), 1 mgm./kgm. It should be noted that the spasmogenic dosage of pilocarpine HCl employed produced some vasodepression and that part of the pressor effect observed after the spasmolytics was probably due to an anticholinergic action. All of these agents produce slight pressor effects in the anesthetized dog (see below).

Since these rabbits were anesthetized, no evidence of central nervous system stimulation was obtainable. However, if any significant stimulant effect had occurred as results after application of such agents as amphetamine sulfate or metrazol, definite analepsis would have been observable. No deepening of anesthesia was noted, indicating that no marked central nervous system depression resulted.

Dogs. The *duration of colonic spasmolysis* after intravenous and subcutaneous dosage of some of the spasmolytics is shown in table 1. Figures 1 and 2 are representative of experiments in which the spasmolytics were administered intravenously and subcutaneously.

Cardiac rate was increased by β-diethylaminoethyl phenyl-α-thienylglycolate, 1 mgm./kgm. given subcutaneously in morphinized and untreated dogs. Mean control cardiac rate for the morphinized dogs was 60 with extremes of from 48 to 72 beats per minute; for the untreated dogs it was 110 with extremes of from 93 to 138 beats per minute. The observed percentage changes from the mean control after subcutaneous administration were as follows: in morphinized

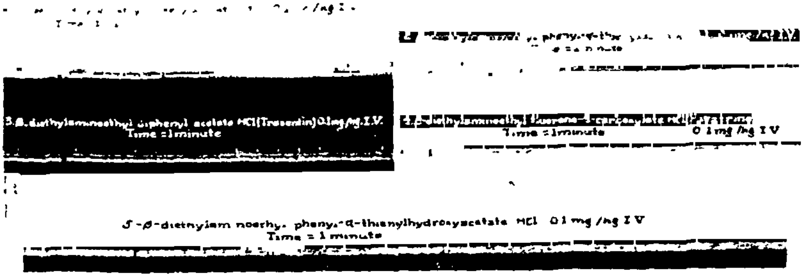

FIG. 1 Colonic activity, dog ♂ morphine sulfate 5 mgm./kgm. i.v. Effects of intravenous injection of spasmolytics on colonic activity of the dog 60–90 minutes after spasmogenic dosage of morphine sulfate. Interruption of base line indicates application of spasmolytic agent.

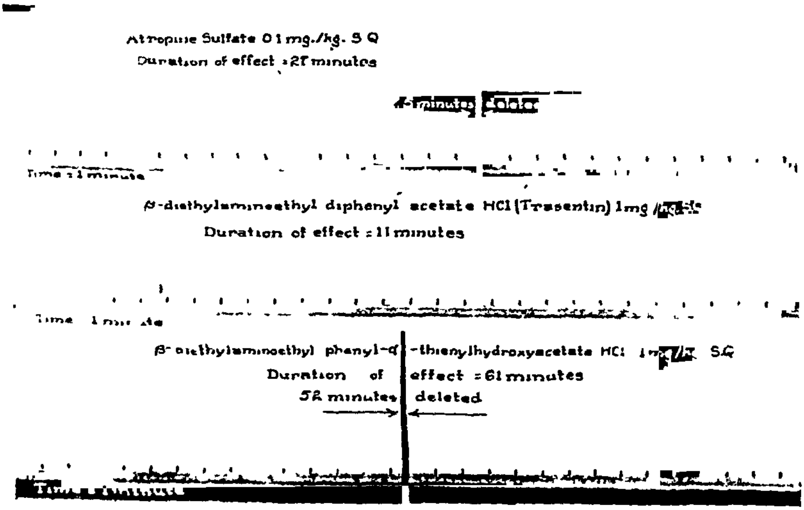

FIG. 2 Colonic activity, morphine sulfate 5 mgm./kgm. s.q. dog ♂. Effect of subcutaneous injection of spasmolytics on colonic activity of the dog 60–90 minutes after spasmogenic dosage of morphine sulfate. Interruption of base line indicates application of spasmolytic agent.

dogs from 140 to 254, and in untreated dogs from 97 to 152. The same untreated dogs on another day received β-diethylaminoethyl phenyl-α-thienylacetate HCl, 1 mgm./kgm. subcutaneously and showed slight elevations in cardiac rate. Percentage changes from their control rate (see above) were from 12 to 15.

Mean blood pressure in dogs anesthetized with morphine sulfate and sodium pentobarbital was generally elevated by small but appreciable amounts. Elevations after intravenous doses of the spasmolytics were as follows: From 0 to 22 mm. Hg above controls averaging 121 mm. Hg by β-diethylaminoethyl phenyl-α-thienyl-glycolate HCl, 0.1 mgm./kgm.; from 2 to 24 mm. Hg above controls averaging 118 mm. Hg by β-diethylaminoethyl phenyl-α-thienylacetate HCl, 1 mgm./kgm.; from 6 to 20 mm. Hg above controls averaging 123 mm. Hg by β-diethylaminoethyl diphenylacetate HCl (trasentin), 1 mgm./kgm.; from 10 to 12 mm. Hg above controls averaging 115 mm. Hg by β-diethylaminoethyl fluorene-9-carboxylate HCl (pavatrine), 1 mgm./kgm.; and from 0 to 14 mm. Hg above controls averaging 116 mm. Hg by atropine sulfate, 0.1 mgm./kgm.

Possible evidence of *central nervous system stimulation or depression* was obtained in unanesthetized dogs which received β-diethylaminoethyl phenyl-α-thienylglycolate HCl, 1 mgm./kgm. subcutaneously. Dogs on an animal board or tied on a short tether showed evidence of marked excitement by barking loudly and forcefully attempting to overcome their restriction. This could be considered evidence of central nervous system stimulation if it were not for the fact that when these animals were not restricted they exhibited ataxia when allowed to walk about on the laboratory floor. It is very likely that what appeared to be central nervous system stimulation when the dog was restricted was only evidence of removal of higher cerebral center inhibition since when the animal was released the only evidences of central nervous system action was cortical depression (ataxia). Similar phenomena have been observed in the dog after subanesthetic dosage of sodium pentothal or after scopolamine HBr, 1 mgm./kgm., intravenously (11). β-diethyl-aminoethyl phenyl-α-thienylacetate HCl, 1 mgm./kgm., subcutaneously in these same dogs on another occasion did not produce any observable changes in their activity. Since dogs showed this marked central nervous system action from β-diethylaminoethyl phenyl-α-thienylglycolate HCl and since it appeared to be a highly potent spasmolytic agent in this species, more intensive studies were made in the monkey to ascertain if any species difference existed.

Monkeys. The duration of colonic spasmolysis after subcutaneous dosage of the four spasmolytics investigated in this species is summarized in table 1. Representative records are presented in Figure 3 and give an indication of the variation in response to two different doses of β-diethylaminoethyl phenyl-α-thienylglycolate HCl.

The cardiac rate was increased by all of the agents but marked differences in response from that of the dog were noted with β-diethylaminoethyl phenyl-α-thienylglycolate HCl. All monkey data were obtained after the spasmogenic dose of morphine sulfate had been administered. Mean control cardiac rate for the group was 230 with extremes of from 214 to 250 beats per minute. The percentage changes from the mean control after subcutaneous administration were as follows: From 0 to 10 after β-diethylaminoethyl phenyl-α-thienylglycolate HCl, 1 mgm./kgm.; from 0 to 12.5 after β-diethylaminoethyl fluorene-9-carboxylate HCl (pavatrine), 1 mgm./kgm.; from 0 to 14 after diethylaminoethyl diphenylacetate HCl (trasentin), 1 mgm./kgm.; and from 5 to 70 after atropine sulfate, 0.1 mg./kgm.

Central nervous system effects did not appear in any of the monkeys which received 1 mgm./kgm. of β-diethylaminoethyl phenyl-α-thienylglycolate HCl.

TABLE I

Duration of spasmolysis in different species expressed in minutes

DRUGS	DOSE PER KGM		RABBIT, I.V.		DOG (COLON)		MONKEY (COLON) S.Q.
	Mgm	mEq × 10^{-3}	Ileum	Colon	I.V.	S.Q.	
β-diethylaminoethyl phenyl α-thienylacetate HCl	0.1	0.282			6		
	1.0	2.82	2–6	2–3			
β-diethylaminoethyl phenol α-thienylglycolate HCl	0.1	0.270			8–24		
	1.0	2.70	17–24	5–22		15–135	19–113
β-piperidinoethyl α methyl p-xenylacetate HCl	0.1	0.267			sl		
	1.0	2.67	2	1–4			
β-diethylaminoethyl diphenylacetate HCl (trasentin)	0.1	0.287			6–8		
	1.0	2.87	2–4	2–9		sl	0
β-diethylaminoethyl fluorene 9-carboxylate HCl (pavatrine)	0.1	0.289			sl –10		
	1.0	2.89					inc to 0
Atropine sulfate	0.1	0.288			sl –50	0–75	58–240

Blank spaces = drug not administered, 0 = no decrease in colonic activity, sl. = decrease in amplitude of contraction or arrest of 1 segment for 1–20 minutes, inc = increase in amplitude 5–15 minutes

One monkey was selected for a prolonged administration experiment. This animal received daily dosages starting at 1 mgm./kgm. intramuscularly, which were increased geometrically until death occurred. The following is a summary of effects produced in this animal. With 1, 2 and 4 mgm./kgm. slight depression occurred in from 10 to 20 minutes after administration, which disappeared within one hour. No appreciable mydriasis or cycloplegia was observed.

With 8 mgm./kgm. within 20 minutes slight incoordination occurred when grasping for food, decrease in normally agile response to prodding. No appreciable mydriasis or cycloplegia was observed.

With 16 mgm./kgm. within 20 minutes animal was depressed and unable to extend arms for grasping food. Ataxia, incomplete mydriasis and cycloplegia were present. Prodding no longer caused the animal to climb about the cage. Recovery was complete in two hours.

With 32 mgm./kgm. within 10 minutes animal was unable to walk, but able to remain erect. Complete mydriasis and cycloplegia were present. Acute effects disappeared within one hour, but slight central depression (lassitude) was noted for one hour longer.

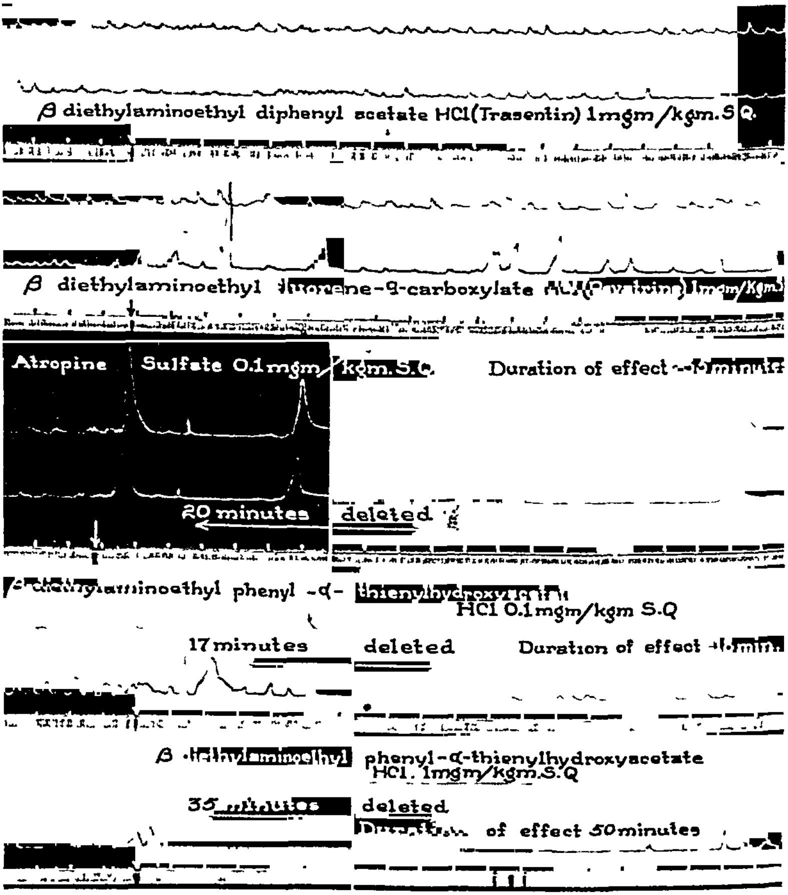

FIG. 3. Colonic activity, monkey ♀ 4 mgm./kgm. morphine sulfate s.q. Time, 1 minute. Effect of subcutaneous injection of spasmolytics on colonic activity of the monkey (Macaqus rhesus) 60-90 minutes after spasmogenic dosage of morphine sulfate. Arrow and interruption of base line indicate application of spasmolytic agent. Disregard interruptions of base line in 4th record which are not accompanied by arrows.

With 64 mgm./kgm. within 10 minutes animal was lying on bottom of cage, then clonic convulsions were initiated. Convulsions continued for 30 minutes until death, which appeared to be due to respiratory depression.

There is some indication from this animal that the safe dose range for monkeys is between 1 and 32 mgm./kgm. intramuscularly. In all probability, the lethal subcutaneous dosage will not be lower and, if anything, will be higher.

COMMENT. In evaluating agents as gastro-enteric spasmolytics, careful consideration should be given to any appreciable action on other autonomic neuro-effectors and on the central nervous system. On the basis of the data presented from the various species investigated, it is evident that the dog is markedly susceptible to what may be considered untoward actions. While such effects might be expected to occur in man, it is recognized clinically (12) that the commonly used agent, scopolamine, does not produce such action unless the individual is subjected to a severe painful stimulus. Since the effects which were observed in the monkey indicate that this species is relatively resistant to some of the peripheral autonomic and to the central nervous system action of β-diethylaminoethyl phenyl-α-thienylglycolate HCl, it is possible that the same might be true in man. Preliminary observations in man given 0.04 mgm./kgm. orally indicate that no unusual untoward effects occur with this derivative at this dose level.

BIOCHEMORPHOLOGY. Substitution of thiophene for one of the benzene nuclei in β-diethylaminoethyl diphenylacetate HCl (trasentin) does not cause any marked alteration in spasmolytic properties in (a) the intact rabbit colon or ileum made spastic with pilocarpine or (b) the dog colon rendered hyperactive with morphine. Introduction of an hydroxyl group in the acetic acid portion of the β-diethylaminoethyl phenyl-α-thienylacetate markedly increases spasmolytic activity in these two species. This is in agreement with the findings of Lands and Nash (9) on the isolated rabbit colon and intact rabbit jejunum. Substitution of an α-thienylglycolate group for phenylacetate in trasentin has increased the anti-morphine action of this agent on the dog and monkey colon, *in vivo.*

SUMMARY

β-diethylaminoethyl phenyl-α-thienylglycolate HCl, in comparison with other related compounds[8, 9]

(1) Effectively opposes the spasmogenic action of:
  A. Pilocarpine on the intact colon and ileum of the anesthetized rabbit.
  B. Morphine on the intact colon of the monkey and dog.

(2) Produces marked cardiac acceleration in the morphine treated and untreated dog; in the monkey cardiac acceleration is not very marked and is less than after the administration of $\frac{1}{10}$ the dose of atropine sulfate calculated on a weight basis.

(3) Produces signs of central nervous system activity resembling delirium in the dog but not in the monkey or rabbit when employed in spasmolytic dosage.

Acknowledgment. Grateful acknowledgment is made to Dr. Albert Raymond of G. D. Searle & Co. for a generous supply of pavatrine and to Dr. M. L. Moore and co-workers of Frederick Stearns & Co. for the new agents utilized in this study.

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STUDIES ON THE PHARMACOLOGY OF DDT (2,2 BIS-(PARACHLORPHENYL)-1,1,1 TRICHLOROETHANE)

I. The Acute Toxicity of DDT Following Intravenous Injection in Mammals with Observations on the Treatment of Acute DDT Poisoning

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Knowledge of the toxicity and actions of DDT in mammalian species is essential to define the incidental hazards associated with widespread dissemination of this unique insecticide. Moreover, a knowledge of the pharmacological actions of DDT in species other than insects may contribute to an understanding of the fundamental mechanism of action of the agent as an insecticide. Finally, isolated cases of DDT poisoning are bound to occur and it is essential that an effective method of treatment be made available.

Studies on the acute and chronic toxicity of DDT have already been reported (1, 2, 3, 4, 5). The fact that DDT is insoluble in water and other vehicles suitable for intravenous administration has limited these investigations largely to observations on the effects of absorption from the gastro-intestinal tract, respiratory tract, and skin. Although these routes are ideally suited for chronic studies, acute responses vary greatly between species and have also proved inconsistent in a given species. This particularly applies following oral administration, the only route available for the study of acute toxicity in most species. Not only has the enforced use of the gastro-intestinal route of administration obscured the absolute toxicity of DDT but it has also rendered difficult studies on routes of excretion, and treatment of acute poisoning.

Because of the above facts a preparation of DDT suitable for intravenous administration was sought. A solution of DDT in peanut oil can be emulsified in isotonic saline containing 1 per cent lecithin. Such an emulsion appears to possess no pharmacological properties other than those that can be attributed to the DDT. Intravenous injections of the emulsion have yielded information on the absolute toxicity of DDT, the time of onset of action of DDT, the possible role of intermediaries of DDT in the manifestation of toxic action and the duration of action of DDT. Moreover, they have also provided a more precise technic for the determination of the efficacy of antidotal procedures in those species which respond capriciously to the oral route of administration.

PROCEDURE. A. *Preparation of emulsion* The DDT-emulsion is most conveniently prepared from two stock solutions a 10 per cent solution of DDT[3] in peanut oil; a solution

[1] 1st Lt, SnC, AUS

[2] Major, SnC, AUS

[3] A purified preparation of DDT, M.P. 108.6–109.5°C, was used in all emulsions

of purified soy-bean lecithin[4] in 0.9 per cent NaCl (1.0 gram of lecithin to 90 ml. of saline solution). The two solutions are mixed in the proportion of one part by volume of the oily solution of DDT and 9 parts by volume of the lecithin solution, shaken thoroughly, and emulsified. The final solutions thus contains 10 mg. of DDT per ml. Emulsification is conveniently accomplished by the use of a manually operated homogenizer. Six successive passages through the homogenizer result in a solution of milky appearance containing oil-droplets which are for the most part less than two red-cell diameters in size. Relatively few droplets as large as 20 to 25 microns in diameter are present. The DDT remains dissolved in the oil-phase of the emulsion. Emulsions should be used immediately or re-homogenized if allowed to stand.

B. Technic of intravenous administration. Dogs, rats, rabbits and monkeys (*Macaca mulatta*) exhibit no immediate untoward reaction to the intravenous administration of freshly prepared emulsions regardless of the rate of injection. In the case of the cat, however, rapid intravenous injection causes stasis in the pulmonary circulation resulting in immediate death. This can be avoided by reducing the rate of injection to approximately 2 to 3 ml. per minute.

EXPERIMENTAL RESULTS. *A. Acute toxicity.* Studies of the acute, intravenous toxicity of DDT have been made in rats, rabbits, cats, dogs and monkeys. Although they respond in the same general manner, species differences are sufficiently marked to warrant individual discussion.

Rats. Rats respond in a fairly uniform manner to acutely lethal, oral doses of DDT. Data on the oral toxicity of a 10 per cent solution of DDT in peanut oil as observed in this laboratory are presented in table 1. They are in general agreement with those reported by Woodward, Nelson and Calvery (2) and Smith and Stohlman (1, 4). Following the oral administration of 300 mgm./kgm. of DDT to rats there is a latent period of approximately two hours. The animals then develop a generalized muscular tremor which increases in intensity to the stage where purposeful movements can no longer be initiated. Episodes of clonic convulsions may occasionally occur, but in the case of the rat tremors are so pronounced that the convulsive episodes are difficult to discern. The great majority of deaths occur between 5 and 24 hours, death resulting from exhaustion and ultimate central depression. Surviving rats still show a marked tremor after 24 hours which gradually abates during the subsequent 24 hours.

If the route of administration of DDT is changed from oral to intravenous, toxicity is markedly increased (*Table 2*) and the latent period before the onset of symptoms is greatly reduced. Following intravenous injection, with the more rapid development of fulminating action, it is possible to discern an orderly sequence of neuromuscular involvement. In response to an intravenous dose of 50 mgm./kgm. of DDT (LD_{94}) there is a latent period of approximately 5 minutes, following which blepharospasm and twitching of the ears and vibrissae can be discerned. Shortly thereafter a fine tremor of the muscles of the head and neck can be noticed. The tremor progresses caudally, meanwhile increasing in intensity. Within 30 minutes it has reached its full development. Over 50 per cent of the animals die within 3½ hours. Survivors are symptom-free after 18 to 24 hours.

Cats. The response of cats to the oral administration of DDT is capricious.

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[4] Lexinol C, American Lecithin Co.

One animal may be relatively unaffected by a dose of 500 mgm./kgm. while another may succumb to one-half this amount despite all attempts to keep conditions as constant as possible. In table 1 are presented scattered data on the oral toxicity of DDT in cats. Symptoms appear only after a delay of one to several hours, depending on dosage, and are greatly prolonged. They resemble those to be described for the intravenous route of administration.

The marked individual variation in susceptibility to DDT is not apparent following intravenous administration. The toxicity data are presented in table 2. Immediately following the intravenous administration of 50 to 60 mgm./kgm. of DDT, cats appear normal provided the emulsion was injected slowly. However, within 5 to 10 minutes they become restless and apprehensive and show the earliest signs of intoxication, namely, blepharospasm and twitching of the ears and vibrissae. Following this, tremor of the facial muscles may be discerned. The tremor spreads over the entire body in the same manner as described for rats. Within 20 to 30 minutes the tremors are general and severe, and purposeful movements are difficult. At this time the animals exhibit frequent tonic extensor thrusts of all four legs of very brief duration. They evidence excitement and the pupils are widely dilated. The first true convulsive episode usually occurs 30 to 40 minutes after injection. It is usually initiated by sudden opisthotonus with the animals falling on their sides. This is immediately followed by severe clonic and tonic movements of the extremities and marked pilo-motor activity. The initial convulsions are of brief duration and are followed by periods during which only the tremor is evident. During this time the animals recover sufficiently to regain an upright position. On attempting to walk they show a marked dysmetria and enhancement of the tremor. They also become greatly agitated by noxious stimuli and appear unable to respond in a purposeful manner or localize the sites of such stimuli. As intoxica-

TABLE 1

Toxicity of DDT following oral administration

SPECIES	DOSAGE	MORTALITY
	mgm./kgm.	
Rat	200	6/10
	250	22/25
	300	99/110
	400	10/10
	500	10/10
Cat	200	0/3
	250	1/4
	300	1/3
	400	2/3
	450	0/1
	500	5/8
	600	1/2

tion advances, the periods between seizures become progressively shorter. In the final stages convulsive activity is almost continuous although weakened in intensity. Eventually the animals become severely depressed and respiratory failure occurs within 2 to 5 hours after injection. The occasional animals which recover from these large doses of DDT are free of symptoms within 18 hours.

Approximately 20 per cent of cats receiving 60 to 75 mgm./kgm. of DDT, intravenously, succumb suddenly, usually during the first but possibly during a subsequent convulsive episode. Death in these instances is due to ventricular fibrillation.

TABLE 2

Toxicity of DDT following intravenous administration

SPECIES	DOSAGE	MORTALITY
	mgm./kgm.	
Rat	30	1/10
	40	4/20
	50	31/33
	60	12/14
Rabbit	25	0/5
	35	0/4
	50	4/4
	75	8/8
Cat	25	0/4
	40	4/4
	50	12/16
	60	13/17
	75	3/3
Dog	25	0/3
	50	0/4
	60	0/2
	75	7/7
Monkey	40	0/1
	50	1/2
	60	1/2
	75	2/2

Rabbits. The data on the toxicity of DDT administered intravenously to rabbits, are presented in table 2. This species exhibits the same order of susceptibility as do rats and cats. The symptoms resemble those seen in cats with the exception that depression occurs earlier. A small percentage of rabbits also dies of ventricular fibrillation.

Dogs. Dogs are unpredictable in their response to the oral administration of DDT, some animals surviving single doses as high as 500 mgm./kgm. Their susceptibility to DDT administered intravenously is somewhat less than that of the species already discussed, but is in the same general range. Limited data are presented in table 2. Following the intravenous administration of 75 mgm./kgm. of DDT (LD100) the sequence of events resembles that described for cats. However, 5 out of 7 animals succumbed to ventricular fibrillation during the first convulsive episode, which usually occurred within 30 minutes after injection. The two dogs which proved resistant to ventricular fibrillation exhibited a prolonged period of intermittent convulsive activity. The earlier convulsive episodes were characterized by an initial tonic phase followed by clonic activity. Clonic activity terminated abruptly. Between convulsive episodes, tremor and occasional extensor thrusts were evident. In both dogs emesis occurred following the first convulsion. Convulsive episodes occurred with increasing frequency until the animals exhibited almost continuous activity. This was characterized by frequent bursts of clonic running movements and punctuated occasionally by tonic extension and flexion of the fore and hind legs. Within 4 hours the animals were depressed, death ultimately resulting from exhaustion and depression. Although doses of 50 and 60 mgm./kgm. were not fatal, they caused severe convulsions.

Monkeys. Limited data on the intravenous toxicity of DDT in monkeys are presented in table 2. Following the intravenous administration of 75 mgm./kgm. there is an asymptomatic period of approximately 5 minutes following which a mild fibrillary tremor in the head region becomes apparent. This tremor progressively descends to involve the forelegs, trunk, and hind legs. Within 15 minutes the animals exhibit a gross static tremor which is accentuated by purposeful movement. Blepharospasm is marked. The first convulsion usually occurs within 20 minutes. It is manifest by a tonic phase characterized by flexion and opisthotonus and followed by simultaneous, repeated extensor thrusts of all limbs. The episode terminates abruptly, leaving the animal depressed and almost free of tremor. The monkey rapidly recovers from early convulsive seizures, recovery bringing on a return of the tremor. Convulsions recur with almost clock-like regularity for a period of several hours, following which the interval between convulsions shortens until the animal shows almost continuous clonic activity. Eventually depression occurs and death results from respiratory failure. Animals receiving sub-lethal doses of DDT are symptom-free after 18 hours despite the occurrence of severe convulsions. Ventricular fibrillation is a common occurrence in monkeys receiving high intravenous doses of DDT. However, in this species spontaneous reversion to an organized beat usually occurs.

B. *Treatment of acute poisoning.* Possible therapeutic agents for the control of neurological manifestations of DDT intoxication were investigated in acutely poisoned rats, cats dogs and monkeys. The drugs chosen have in common sedative, depressant, or anticonvulsant properties. In evaluating their therapeutic action attention was directed not only to the prevention of mortality in animals which received lethal doses of DDT but also to the extent to which the agent under study ameliorated such signs of intoxication as gross tremor and convulsions.

The results obtained are presented in tables 3 and 4. Of the various agents employed, phenobarbital was by far the most outstanding. Not only was it effective in preventing the death of animals but also tremor and convulsions were controlled with doses that were well below the anesthetic level. Symptoms were more readily controlled in dogs and cats than in monkeys. In the last-mentioned species close to full anesthetic doses were required before tremors completely disappeared.

The effects of the other agents may be summarized briefly. Magnesium sulfate effectively prevented tremors and convulsions for a brief period but its action was too evanescent to reduce mortality. Sodium bromide, in view of the large doses employed, the high mortality of treated animals and the absence of symptomatic relief, must be considered an ineffective agent. Urethane reduced mortality but symptoms were only alleviated with full anesthetic doses. Likewise, sodium barbital and sodium pentobarbital controlled the symptoms only when given in full anesthetic doses. Such doses did not effect a dramatic reduction in mortality. Dilantin when administered prophylactically reduced the lethality of DDT in rats despite the fact that there was not a significant amelioration in the symptomatology of the animals. The agent proved to be ineffective in cats, however.

DISCUSSION. Following the intravenous administration of DDT the symptoms observed in the various species studied closely resemble those which result from oral administration. However, three significant differences are worthy of comment: the lethal dose by the intravenous route is approximately only one-tenth that by the oral route; the onset of symptoms is only delayed for five minutes; the duration of symptoms is significantly shortened.

The marked difference between the oral and intravenous lethal dose of DDT can best be attributed to slow and incomplete absorption from the intestinal tract. Another possible explanation for the relatively low oral toxicity might be a rapid detoxification when absorption occurs through the portal circulation. However, studies on the chronic toxicity of DDT (1, 2, 3, 4) which have indicated that small daily oral doses cause a cumulative effect do not support the concept that rapid detoxification occurs. Similarly, the fact that symptoms following the oral administration of DDT are much more prolonged than when the agent is administered intravenously also may be explained by a relatively long period of absorption.

The delay of onset of the symptoms of DDT intoxication following its oral administration has been the subject of speculation. So prolonged is the latent period that the possibility of a product of the intermediary metabolism of DDT being the cause of toxic symptoms has been entertained. Following the intravenous administration of DDT the latent period between administration and onset of action is so reduced as to discount, to a large degree, the possibility of a toxic intermediary necessarily being the cause of the symptoms of poisoning. In order to test this point further, a series of experiments was performed on eviscerated cats. Under light ether anesthesia, the gastro-intestinal tract from the esophagus to the rectum, and the spleen were removed. Hepatic function was eliminated by ligation of the hepatic artery and portal vein. Following the

TABLE 3

*Treatment of rats following acute oral intoxication with DDT**

TREATMENT			MORTALITY
Therapeutic Agent	Total Dose	Time of Treatment	
	mgm./kgm.	*hours*	
None			99/110
$MgSO_4 \cdot 7H_2O$	600	3–5.5	9/10
	1200	2–6	10/10
NaBr	1000	2.5	6/10
Urethane (ethyl carbamate)	500	3–5	5/10
Barbital Na	200	3	8/10
	250	3	6/8
	300	3	10/10
Phenobarbital Na	50	3	7/10
	150	3–5	4/10
	150	3	1/10

* 300 mgm./kgm.

TABLE 4

Treatment of acute intravenous DDT poisoning in various species

SPECIES	DDT DOSAGE	TREATMENT				MORTALITY
		Therapeutic Agent	Time of Administration	Route of Administration	Total Dosage	
	mgm./kgm.		*hours*		*mgm./kgm.*	
Rat	60	Dilantin, sodium	−5	oral	1000	0/5
			−5	oral	500	0/5
			−4.5	oral	250	1/5
			−4	oral	100	1/5
Cat	60	Dilantin, sodium	−4	oral	250	4/5
	60	Urethane (ethyl carbamate)	+½ to +3½	intraperitoneal	500	2/3
					750	0/4
	50	Pentobarbital sodium	+½ to +4½	intraperitoneal	65	6/9
	50 to 60	Phenobarbital sodium	+½	intraperitoneal	50	0/11
					75	2/12
Dog	75	Phenobarbital sodium	+½	intraperitoneal	40 to 50	0/6
Monkey	75	Phenobarbital sodium	+½	intraperitoneal	p.r.n.	1/6

operation the animals received an intraperitoneal injection of 50 ml./kg. of a solution of 5.5 per cent glucose and 0.9 per cent NaCl. One to two hours later when the animals were ambulatory and completely recovered from the effects of the anesthetic they received, by intravenous injection, 50 mg./kg. of DDT in the form of emulsion, the dose being based on the post-operative weight. Within 5 to 10 minutes the typical course of fatal intoxication began. The animals were dead within 90 to 105 minutes. Eviscerated controls receiving an emulsion of oil and NaCl-lecithin solution containing no DDT remained unaffected. Thus, in eviscerated and functionally hepatectomized animals in the absence of organs primarily concerned with the metabolic transformation of foreign substances, the time of onset and subsequent course of DDT intoxication is not altered. The possibility that such a transformation may occur in the nervous system still remains.

It is evident from the observations on various species that anticonvulsants and central depressants exert a physiological antagonism to the heightened central activity produced by DDT. Not all central depressants are equally effective. Of the limited number studied, phenobarbital appears to be outstanding in that tremor and convulsions are more completely controlled with doses producing a minimal degree of depression.

The specific antagonistic action of phenobarbital is in keeping with the finding that studies on the localization of the central action of DDT indicate that the motor cortex is prominantly involved (6). The specific depressant action of phenobarbital on the motor cortex in distinction to other barbiturates has been pointed out by Keller and Fulton (7).

Chronic DDT poisoning is characterized by degenerative changes in the liver and other organs (8, 9) and a tendency for the neurological symptoms to become persistent, even after DDT is withdrawn. In such animals degenerative lesions of the central nervous system can be demonstrated (10). It is of interest to note, therefore, that on the basis of casual observation of general behavior, appetite, etc., none of the above animals showed any residual effects from a single large dose of DDT. Apparently the toxic effects of DDT on cells which are known to be susceptible to injury by chlorinated hydrocarbons are negligible when compared with the acute actions of this insecticide on the central nervous system.

SUMMARY AND CONCLUSIONS

The preparation of an emulsion in which saline is the continuous phase, a solution of DDT in peanut oil the dispersed phase, and lecithin the emulsifying agent is described. The emulsion without DDT possesses no significant pharmacological action.

The toxicity of DDT administered intravenously in the form of emulsion is of the same order for rats, rabbits, cats, dogs and monkeys. The intravenous lethal dose is approximately one-tenth the oral lethal dose.

Following the intravenous administration of DDT, symptoms of intoxication are evident within 5 to 10 minutes. This is in contrast to the latent period of several hours which follows oral administration.

The symptoms of acute DDT intoxication in mammals consist predominantly of muscle tremors and tonic and clonic convulsions. These symptoms are probably caused by DDT itself rather than a metabolic transformation product.

Acute DDT intoxication can cause death by two mechanisms, namely, central excitation followed by depression and respiratory failure, and ventricular fibrillation. Rats, and the majority of rabbits, cats and monkeys die of the central effects. The majority of dogs succumb to ventricular fibrillation.

The effectiveness of a series of central nervous system depressants in the control of acute DDT intoxication in rats, cats, dogs and monkeys has been studied. Of the various agents evaluated, phenobarbital is outstanding in that it controls the characteristic tremor and convulsions produced by DDT in doses that cause a minimal degree of general central depression.

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STUDIES ON THE PHARMACOLOGY OF DDT (2,2, BIS-PARACHLORPHENYL-1,1,1 TRICHLOROETHANE)

II. The Sensitization of the Myocardium to Sympathetic Stimulation during Acute DDT Intoxication

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During the course of investigation on the symptoms of acute poisoning which follow the intravenous administration of emulsions of DDT (1), it became apparent that a small percentage of rabbits and cats and the majority of dogs died atypically early in the course of poisoning. In the case of dogs, death occurred precipitously during the first convulsive episode. Inasmuch as the severity of the convulsion was insufficient to account for death, experiments to elucidate the underlying mechanism were initiated. It is known that hydrocarbons and halogenated hydrocarbons are capable of sensitizing the myocardium in such a manner that small amounts of epinephrine or sympathin can produce ventricular fibrillation (2). What is more, sympathetic discharge as evidenced by extreme mydriasis and marked pilo-motor activity is a prominent feature of a DDT-induced convulsion (1). Attention was therefore directed to the possibility that sudden death following the administration of DDT was due to ventricular fibrillation of a heart sensitized by a chlorinated hydrocarbon and subjected to excessive sympathetic activity.

Experimental procedure and results. Dogs and monkeys (*Macaca mulatta*) were employed as experimental subjects. Two types of experiments were performed. In the first type the vulnerability of the DDT-sensitized heart to epinephrine was determined. In the other, the effect of intrinsically produced sympathin on the sensitized myocardium was demonstrated.

For the first type of experiment only dogs were employed. Animals weighing approximately 15 kgm. were anesthetized by the intraperitoneal injection of 100 to 125 mgm./kgm. of sodium phenobarbital. They then received, by intravenous injection, 75 to 100 mgm./kgm. of DDT in the form of a 1 per cent emulsion (1). At intervals thereafter they were challenged by the intravenous injection of varying amounts of epinephrine. As controls, animals receiving comparable volumes of oil-saline emulsion containing no DDT were challenged with varying doses of epinephrine. The cardiac response was followed either by auscultation or the ECG. The results may be summarized as follows: Fibrillation was induced in 2 of 3 dogs which had received 75 mgm./kgm. of DDT intravenously and in 6 of 8 dogs which had received 100 mgm./kgm. of

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[1] 1st Lt., SnC, AUS.

[2] Major, SnC, AUS.

DDT intravenously. Five of the animals succumbed to a challenge dose of 0.01 mgm./kgm. of epinephrine and 1 each to 0.015, 0.02, and 0.04 mgm./kgm. respectively. The challenge dose of epinephrine was injected at varying times ranging from 10 to 60 minutes after the administration of DDT. Of 5 dogs receiving the control emulsion and challenged with 0.04, 0.05, 0.05, 0.1, and 0.1 mgm./kgm. epinephrine, respectively, all survived and showed no electrocardiographic abnormalities other than those which could be expected from those doses of epinephrine.

Four of the animals included in the above data were tested under rigidly controlled conditions deserving of more detailed description. Dogs of similar weight were paired and anesthetized simultaneously with the same dose of phenobarbital. Cannulae were inserted into the femoral veins and attached by rubber tubing to injection burettes. Electrocardiograms (leads 1 and 3) were recorded simultaneously for both animals by means of a 4-channel Grass amplifier and ink recorder. Before receiving emulsion both animals were simultaneously tested for their response to the same challenge dose of epinephrine (0.01 mgm./kgm.). The electrocardiographic response is shown in Figure 1. Following this, emulsions were administered simultaneously by burette at the rate of 0.5 ml./second. One member of the pair received 10 ml./kgm. of an emulsion containing 1 per cent DDT (100 mgm./kgm.) while the control animal received an equivalent amount of emulsion containing no DDT. As can be seen (fig. 1) the intravenous injection of DDT is not without effect on the ECG. During the course of injection characteristic changes in the T-wave occur which usually consist of an inversion of the T-wave in all leads. The rate may also be increased, but in an anesthetized animal ectopic beats are seldom observed. These changes persist over a period of hours. Seven minutes after the completion of the injection of emulsion, both animals were challenged with 0.01 mgm./kgm. of epinephrine. The response of the control animal is no more ominous than that exhibited to the same dose of epinephrine before receiving emulsion. On the other hand, the animal which had received DDT fibrillated promptly. Ten minutes later the control animal received five times the dose of epinephrine that had caused ventricular fibrillation in the animal which had received DDT. This dose of epinephrine caused ventricular tachycardia for a period of 30 seconds, following which a normal sinus rhythm was resumed. As further proof of the sensitizing action of DDT, the control was then injected with 10 ml./kgm. of DDT-emulsion (100 mgm./kgm.) intravenously. Again the characteristic changes in the T-wave were observed. Nine minutes after the completion of the DDT injection, ventricular fibrillation resulted from the intravenous administration of 0.01 mgm./kgm. of epinephrine. In a similar experiment in which paired animals received 7.5 mgm./kgm. of control emulsion and DDT emulsion, respectively, ventricular fibrillation was produced in the experimental animal with a challenge dose of 0.015 mgm./kg. of epinephrine while the control animal showed a typical response to both 0.015 and 0.05 mgm./kgm. of epinephrine. Following this, the control animal received 75 mgm./kgm. of DDT emulsion intravenously, and 20 minutes later fibrillated in response to a challenge dose of 0.01 mgm./kgm. of epinephrine.

Despite the demonstration that the intravenous injection of DDT sensitizes the myocardium to epinephrine it still remained to be shown that events associ-

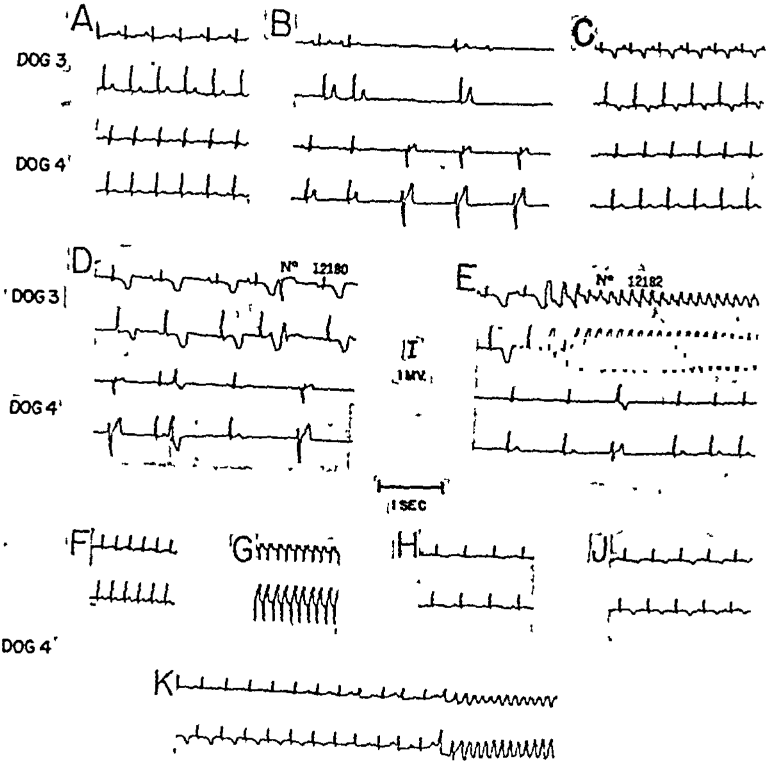

FIG 1 THE INDUCTION OF VENTRICULAR FIBRILLATION BY EPINEPHRINE IN DDT SENSITIZED DOGS

Leads 1 and 3 throughout, 4 channel simultaneous recording Time axis of F and G one-half that of other records A, Control ECG B 20 seconds after the intravenous injection of 0 01 mgm /kgm epinephrine Between B and C dog 3 received 10 ml /kgm of 1 per cent DDT emulsion, Dog 4 received 10 ml /kgm of control emulsion without DDT, both injections given simultaneously over a period of 12 minutes C, 7 minutes after end of DDT injection showing characteristic effects of DDT on T wave D 10 seconds after C and 30 seconds after the intravenous injection of 0 01 mg /kg epinephrine E, 20 seconds later showing onset of ventricular fibrillation in Dog 3 which had received DDT F, Dog 4, 10 minutes after E, and immediately prior to the injection of 0 05 mgm /kgm epinephrine G, 40 seconds later at height of epinephrine action, ventricular tachycardia H, 10 minutes later and immediately prior to the injection of 10 ml /kgm of 1 per cent DDT emulsion J, 9 minutes after the completion of DDT injection K, 20 minutes after the intravenous injection of 0 01 mgm /kgm of epinephrine showing onset of ventricular fibrillation

ated with a DDT-induced convulsion could produce fatal cardiac arhythmias. For this purpose experiments were performed in dogs and monkeys in which simultaneous recordings were made of the EEG and ECG following the administration of DDT. The technic, which was the same in both species, was as follows: under procaine anesthesia a tracheal cannula was inserted and the animal immobilized by the slow intravenous injection of a paralyzant dose of curare (4.0 mgm./kgm. Intocostrin), respiration being maintained artificially. Following this, under local anesthesia to prevent sensory disturbances, the calvarium was exposed. The EEG was recorded by means of a Grass amplifier and ink recorder. The electrodes employed were small brass screws inserted into the calvarium to a depth which caused no injury to the dura. The indifferent electrode was placed on the ear. Simultaneously the ECG was recorded.

A total of 8 experiments was performed in this manner, 6 on dogs and 2 on monkeys. The dogs received either 75 or 100 mgm./kgm. of DDT intravenously in the form of emulsion; the monkeys received 75 mgm./kgm. Three of the dogs died of ventricular fibrillation within 10 minutes after the administration of DDT and before a typical convulsive episode appeared in the EEG. This must be attributed to emotional disturbances resulting from the experimental procedure inasmuch as no dog under general anesthesia has shown severe arrhythmias in response to the doses of DDT employed above. In another dog the administration of DDT resulted in frequent premature systoles, but not until after the onset of the electrical manifestations of convulsive activity did ventricular fibrillation occur (fig. 2, A, B, C). In still another dog the administration of DDT was without significant effect on the ECG until the onset of convulsive activity, as evidenced by the EEG. At this time the first premature systole initiated ventricular fibrillation. (fig. 2, D, E). In the final dog ventricular fibrillation did not occur. This dog exhibited repeated convulsive episodes in the EEG, during which changes in the T waves were the only cardiac reflection of the enhanced central activity.

Of the 2 monkeys studied, one showed a normal sinus rhythm between convulsive episodes and a severe cardiac arhythmia during convulsive episodes. The other monkey was unique in that in this animal a spontaneous return to an organized beat followed ventricular fibrillation. It was thus possible to follow it through a series of convulsive episodes. During each convulsion, short periods of ventricular fibrillation occurred. Between convulsive episodes a normal sinus rhythm prevailed. This continued for 1½ hours, after which convulsions failed to elicit fibrillation but were accompanied by numerous ectopic beats. The course of this monkey is shown in figure 3.

Discussion. From the above experiments it may be concluded that DDT shares with other hydrocarbons and chlorinated hydrocarbons the propensity for sensitizing the myocardium to extrinsic epinephrine or intrinsic sympathin. Although little is known of the distribution of DDT in the body following intravenous administration, it has been shown that by the use of this route of administration typical symptoms of DDT poisoning begin within 5 to 10 minutes and are fully developed within 30 minutes (1).

In the above experiments myocardial sensitization was demonstrable during this time and for several hours thereafter. Assuming an equal distribution of DDT throughout extracellular fluid, a dose of 75 mgm./kgm. would result in a

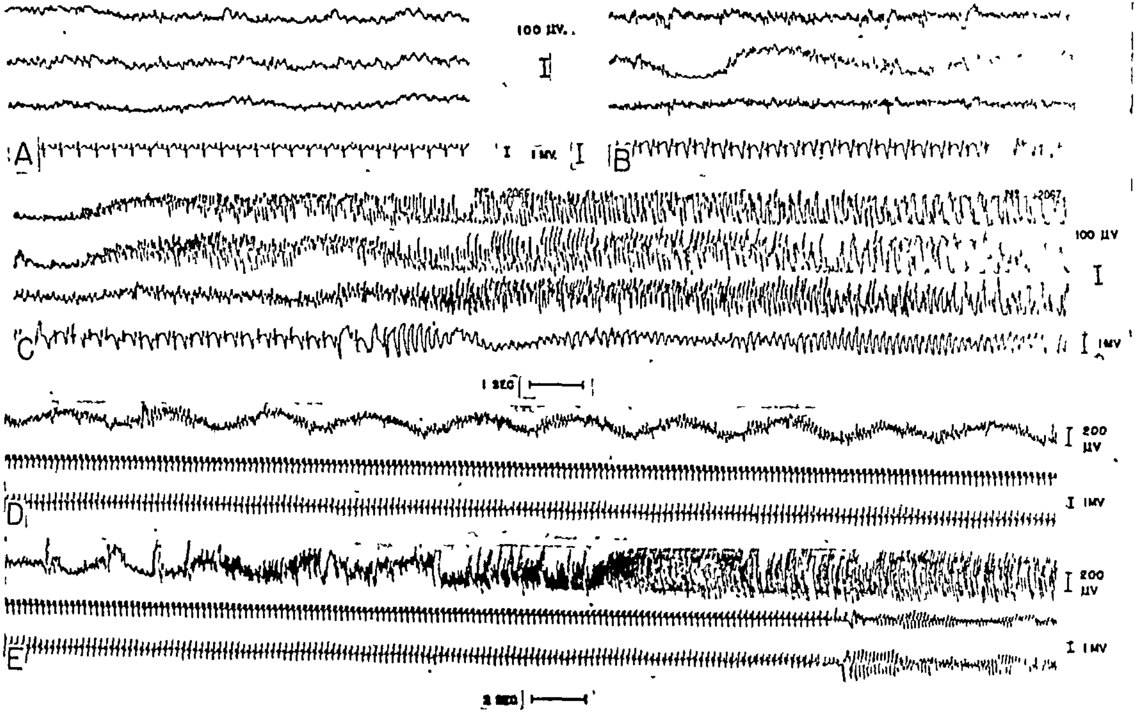

FIG. 2 THE SPONTANEOUS OCCURRENCE OF VENTRICULAR FIBRILLATION IN CURARIZED DOGS DURING A DDT-INDUCED CONVULSION AS MANIFEST BY THE EEG

A, B and C, Dog 2, 16 7 kgm From top to bottom, channels 1, 2 and 3, EEG recorded from screw electrodes in calvarium over parietal lobe, channel 4 ECG, lead 2 A, control record B, 21 minutes after the intravenous injection of 10 ml /kgm of 1 per cent emulsion of DDT showing profound changes in T-wave C, 1 minute later showing the onset of ventricular fibrillation within a few seconds after the beginning of a convulsive episode. D and E, Dog 9, 10 5 kgm. From top to bottom channel 1, EEG from parietal lobe, channel 2 ECG lead 1, channel 3 ECG lead 3 D, 50 minutes after the intravenous injection of 7 5 ml /kgm. of 1 per cent emulsion of DDT Normal sinus rhythm with complete absence of ventricular extra systoles E, 2 minutes later showing the onset of ventricular fibrillation following the first premature systole during a convulsive episode.

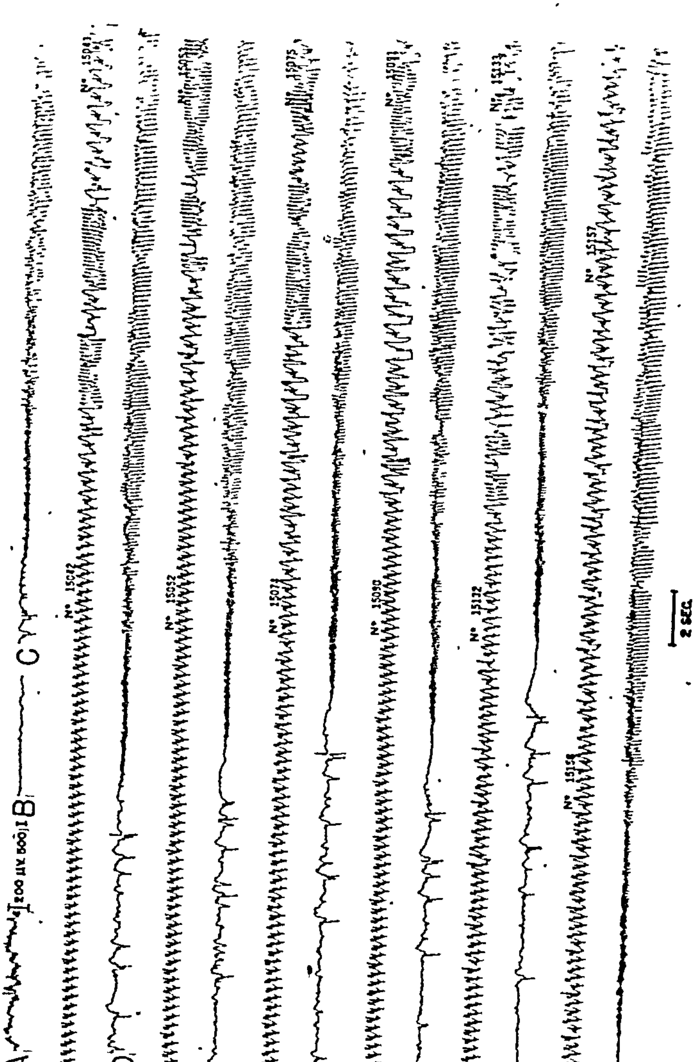

Fig 3 The Occurrence of Spontaneous Ventricular Fibrillation in a Curarized Monkey during Repeated DDT-induced Convulsions as Manifest in the EEG

Top channel, ECG lead 2, bottom channel, EEG recorded from screw electrode in calvarium over area 4. A, control record. B, 65 minutes after the intravenous administration of 7.5 ml./kg. of 1 per cent DDT emulsion. C, 3 minutes later, the onset of a convulsive episode and the spontaneous occurrence of short runs of ventricular fibrillation interspersed with effective systoles. D, 8 minutes later, another convulsive episode during which a change from sinus rhythm to ventricular fibrillation occurs. E, F, G, and H, the same phenomenon recurring at intervals of 3, 9, 6 and 10 minutes, respectively. J, a convulsion characterized by frequent ectopic ventricular beats, but no periods of fibrillation.

concentration of approximately 30 mg. per cent. If distribution throughout the total body water is assumed the concentration in body fluids would be 10 mg. per cent. On this basis, DDT proves to be highly active in sensitizing the myocardium when it is considered that the anesthetic concentration of chloroform in blood is approximately 30 mg. per cent and that of cyclopropane presumably much higher.

It should be emphasized that the animals in the above experiments were completely paralyzed by curare. Thus, no motor manifestations of the convulsant activity of DDT were evident. This precludes the possibility that myocardial anoxia, which might result from the high oxygen demands accompanying a convulsion in a non-curarized animal, could contribute to the onset of fibrillation. Despite the fact that ventricular fibrillation occurred prematurely in some animals and failed to develop in others, the correlation between the electrical activity of the brain and cardiac arhythmias justifies the conclusion that there is a causal relationship between the two. In the unanesthetized animal a DDT-induced convulsion is accompanied by obvious manifestations of sympathetc discharge, i.e., pilo-erection and mydriasis. It is conceivable that this activity results from discharge of the adrenal medulla consequent to the emotional disturbances accompanying the convulsion. However, a more likely explanation is that the autonomic centers of the hypothalamus share in the central stimulatory actions of DDT. Thus, DDT is unique among those compounds sensitizing the myocardium in that it not only sets the stage for ventricular fibrillation, but also, as a result of its central action provides the stimulus necessary for the ultimate onset of fibrillation.

SUMMARY AND CONCLUSIONS

The mechanism whereby DDT causes sudden death by ventricular fibrillation has been investigated. The intravenous injection of 75 to 100 mgm./kgm. of DDT in dogs sensitizes the myocardium so that the intravenous injection of 0.01 mg./kg. of epinephrine results in ventricular fibrillation. Control animals show no evidence of sensitization. In curarized dogs and monkeys convulsive seizures, as evidenced by increased electrical activity of the brain, are accompanied by cardiac arhythmias or ventricular fibrillation. It is concluded that DDT both sensitizes the myocardium and causes sympathetic discharge. The latter may be reflex, but more probably is the result of direct hypothalamic stimulation. It is this dual action which is responsible for the onset of ventricular fibrillation.

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THE ANTI-HISTAMINE PROPERTIES OF BENADRYL, β-DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE

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The name Benadryl has been given to β-dimethylaminoethyl benzhydryl ether hydrochloride, one of several homologous ethers capable of alleviating bronchoconstriction induced in guinea pigs by administration of histamine (1) or by its release in anaphylaxis (2). Experiments herein reported were conducted to determine whether Benadryl antagonizes diverse actions of histamine on several tissues, viz., stimulation of secretory cells, relaxation of vascular smooth muscle, and contraction of intestinal smooth muscle. Furthermore, it was deemed important to determine whether Benadryl antagonizes histamine in a specific manner or whether the effects of other depressor and/or spasmogenic agents such as acetylcholine and barium are altered. Finally, there was the prospect that the data would give some indication regarding the nature or mode of anti-histamine action.

EXPERIMENTAL. *Results.* A. *Prevention of induced intestinal spasm.* Segments of guinea pig ileum were suspended in 100 cc. of aerated Tyrode's solution maintained at a temperature of 38°C. Spasm was induced by histamine diphosphate, 1:12,500,000; barium chloride, 1:10,000; or acetylcholine bromide, 1:50,000,000. With a given spasmogenic agent, several contractions of approximately equal magnitude were elicited. Benadryl, or one of several antispasmodics, was then added to the muscle bath and retained therein for one minute before re-introduction of the standard amount of spasmogenic agent, the contracting effect of which was partially inhibited or annuled. The muscle segment was washed as each contraction became maximal. Following the addition of test drugs, full recovery of muscle sensitivity was always demonstrated before proceeding with the tests. Several compounds, both test drugs and one or two reference antispasmodics, were alternated with each other in each experiment with a given muscle. Various dilutions of each were employed to inhibit spasm by unequal degrees, to match inhibitory effects of two or more drugs and to gradually approach dilutions which would diminish spasm by 75 to 100 per cent. The range of dilutions recorded in table 1 indicate the quantities of the respective drugs which were required to antagonize histamine, barium and acetylcholine. Frequent determination of absolute values for effective dilutions, and numerous comparisons of relative potencies in individual experiments on the same muscle, constitute reasonable assurance that the comparison of drug potency is reliable. It should be pointed out, however, that the duration of drug action (one minute) was arbitrarily chosen and might not permit an expression of true potency for drugs which act slowly.

Results presented in table 1 indicate interesting differences between various antispasmodics. Papaverine is of low potency but practically of equal effectiveness against histamine, barium and acetylcholine. Dilutions of papaverine which inhibited the spasmogenic action of histamine are essentially the same as those reported by Staub (3). Pavatrine and Trasentin proved to be about equally effective in antagonizing histamine and are ten to twelve times more potent than papaverine. Both Trasentin and Pavatrine were markedly superior to papaverine in regard to antagonism of barium and acetylcholine; also, Pavatrine proved to be four or five times more potent that Trasentin in this respect. Atropine exerted moderate anti-histamine action, very weak antagonism of barium, and pronounced antagonism of acetylcholine.

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Of these antispasmodic drugs, only papaverine and atropine were found to be effective in reducing the severity of bronchoconstriction induced by administration of histamine to guinea pigs (1, 4). The activity indexes previously reported (1, 4) and the activity index for a quaternary ammonium derivative of Benadryl are included in table 1 and represent degrees of increased potency over aminophylline which was assigned an activity index of unity.

TABLE 1

Maximum dilutions of antispasmodics which inhibit the spasmogenic effects of histamine, barium and acetylcholine, on isolated guinea pig ileum*

DRUG	HISTAMINE	BARIUM	ACETYLCHOLINE	ANTIASTHMATIC INDEX (CF. REF. 1)
Papaverine HCl	1: 66,000– 100,000	1: 33,000– 50,000	1: 50,000– 66,000	2
Trasentin HCl	1: 833,000– 1,250,000	1: 125,000– 250,000	1: 4,000,000– 6,670,000	0
Pavatrine HCl	1: 833,000– 1,000,000	1: 667,000–1,000,000	1: 25,000,000– 50,000,000	0
Atropine SO_4	1: 500,000– 667,000	1: 5,000– 10,000	1: 100,000,000–200,000,000	3
1) B-Dimethylaminoethyl benzhydryl ether HCl (Benadryl)	1: 25,000,000–50,000,000	1: 66,700– 125,000	1: 2,500,000– 4,000,000	33
2) B-Benzhydryloxyethyl trimethylammonium iodide	1: 10,000,000–20,000,000	1: 40,000– 50,000	1: 8,330,000– 16,600,000	100
3) N-Phenyl-N-ethyl-N'-diethyl ethylene diamine HCl (1571F)	1: 2,000,000– 3,330,000	1: 6,670– 8,330	1: 66,700– 100,000	16

* Drug dilutions within the range recorded diminished spasm by 75 to 100 per cent.

The effects of several anti-histamine compounds on intestinal muscle were compared with the antispasmodics discussed above. Benadryl and a quaternary ammonium derivative, β-benzhydryloxyethyl trimethylammonium iodide, were compared with the Fourneau histamine antagonist, 1571F (3, 5), as well as the antispasmodics. Benadryl was forty to fifty times more potent as an antihistamine agent than Pavatrine and Trasentin, the most effective antispasmodics. It exerted weak antagonism against barium and moderate antagonism against acetylcholine, the latter being one-half to one-tenth that exhibited by Trasentin and Pavatrine. Since low dilutions of Benadryl antagonize barium and acetylcholine, it cannot be regarded as a strictly specific histamine antagonist. However, its specificity against histamine approaches the specificity which atropine displays against acetylcholine.

The quaternary ammonium derivative of Benadryl was approximately one-half as effective as Benadryl against histamine and barium, but four times more potent with respect to antagonism of acetylcholine action on intestinal muscle. Thus, atropine-like qualities were increased at the expense of anti-histamine properties. Nevertheless, this quaternary ammonium compound proved to be definitely superior to Benadryl in reducing histamine-induced bronchoconstriction in guinea pigs, the former being one-hundred and the latter thirty-three times more potent than aminophylline (table 1). Of the two compounds, Benadryl possesses greater potency and more specificity against the spasmogenic effects of histamine on intestinal muscle. Since the comparisons were made on a weight basis it is important to consider that the molecular weight of Benadryl and the quaternary derivative are 291.82 and 397.3, respectively.

The Fourneau histamine antagonist, 1571F, was comparatively impotent in antagonizing barium and acetylcholine. The anti-histamine action was definite but much less than that exerted by the benzhydryl ethers. 1571F possesses appreciable anti-histamine specificity, a fact first reported by Staub (3) who pointed out that 1571F was more specific than 929F and other Fourneau histamine antagonists.

None of the drugs, with dilutions indicated in table 1, depressed the sensitivity of the intestinal muscle for long intervals. However, lower dilutions not only completely eliminated spasm but the residual effects prolonged recovery so that several washings and applications of spasmogenic agents were necessary before normal contractions were again obtainable.

B. *Inhibition of histamine-induced gastric secretion.* The ability of Benadryl to antagonize the effects of histamine on smooth muscle *in vitro* and *in vivo* suggested that it might also diminish the secretogogue action of histamine on glandular cells such as the gastric glands.

Experiments were made on four female dogs weighing 7 to 10 kgm. which were surgically provided with vagal denervated gastric pouches (Heidenhain) several weeks before use. Food was withdrawn 16 to 18 hours before each experiment. The technique and experimental conditions were nearly identical to those described in studies of other histamine antagonists (6, 7). Control data consisted of measurements of the secretory output from the gastric pouches for 75 minutes following histamine stimulation. Two hours later, the animals were treated with Benadryl and after twenty minutes, stimulated with histamine. The secretion obtained during 75 minutes was compared with control values. The secretory stimulus consisted of a subcutaneous injection of 0.5 mgm. (0.5 cc.) of histamine diphosphate. Benadryl was injected subcutaneously in a dose of 10 mgm./kgm. (5 per cent aqueous solution). This dose represents one half to one third of the quantity which produces excitation and tremors. Gastric juice was collected each 15 minutes and the volume recorded. Each sample was then titrated *in toto* for free acid and the total acid, using Topfer's reagent and phenolphthalein, respectively, as indicators.

The data (table 2) reveal that the mean values for volume, total acid and free acid of gastric juice obtained in 12 experiments on 4 dogs treated with Benadryl were 37, 11 and 13 per cent less, respectively, than those obtained following the same histamine stimulus during the control period when no Benadryl was administered. For some unknown reason, secretion was never significantly reduced in one of the four dogs, whereas it was inhibited by 40 to 80 per cent in 8 of 9 experiments with the other three dogs.

There was no evidence of drowsiness, asthenia, excitation, nausea (salivation), vomiting or diarrhea following subcutaneous administration of Benadryl in a dose of 10 mgm./kgm. The dogs invariably possessed good appetites when returned to their cages two hours following drug treatment.

C. *Vascular response to histamine.* The intravenous injection of small quantities of histamine elicits a precipitous, transient fall in blood pressure in the dog. This depressor response to small doses of histamine is alleged to be due to relaxation of arteriolar smooth muscle and capillaries (8, 9). Small doses of acetylcholine act somewhat similarly to elicit depressor responses. Since Benadryl inhibited the contracting effects of histamine and acetylcholine on intestinal muscle it was of interest to determine whether the drug would antagonize the relaxing effects of histamine and acetylcholine on the vascular system. The pressor effect of a standard dose of epinephrine (10γ) was determined repeatedly to test the constancy of vascular responses and to determine whether epinephrine reversal resulted following treatment with Benadryl.

TABLE 2

Effect of benadryl on gastric secretion

NO. OF DOGS	NO. OF EXPTS.	TREATMENT	DIFFERENCE BETWEEN SECRETION WITHOUT AND WITH DRUG TREATMENT								
			Volume of secretion (cc.)			Total HCl secreted (mgm.)			Free HCl secreted (mgm.)		
			Mean	$M_1 - M_2$ ± P.E.*	Per cent change	Mean	$M_1 - M_2$ ± P.E.*	Per cent change	Mean	$M_1 - M_2$ ± P.E.*	Per cent change
4	12	None (A.M.)	10.9			48.4			41.03		
4	12	Benadryl† (P. M.)	6.9	4.0 ±0.97	−36.7	28.72	19.68 ±4.3	−40.6	23.35	17.6 ±3.6	−43.0

* Difference between means considered as significant if greater than four times the probable error (Pearl, 1940).

† 10 mgm./kgm., subcutaneously, 20 min. before the histamine injection in the P.M.

Experiments were made on mongrel dogs (8 to 15 kgm.) anesthetized with phenobarbital sodium administered intraperitoneally. Blood pressure was recorded manometrically from the carotid artery, and respiration by a tambour connected to a tracheal cannula provided with two flutter valves (10) so arranged as to indicate alterations in rate and amplitude. All injections were made into an exposed femoral vein. Figure 1 illustrates the major portion of a typical experiment. In each experiment the depressor response to approximately 10 γ (range, 4 to 15 γ) of histamine was recorded and then a dose of acetylcholine which elicited a comparable depressor effect was selected (2 to 60 γ). The standard dose of epinephrine was alternated with histamine and acetylcholine, the drugs being injected at intervals of 3 to 6 minutes. Three injections of each, i.e., epinephrine hydrochloride, histamine diphosphate, and acetylcholine bromide, were made before injecting Benadryl and again thereafter. Mean responses (table 3) following twelve injections of histamine, acetylcholine, and epinephrine were compared with the mean of a similar number of responses to each agent following intravenous injection with Benadryl or atropine. Four experiments were made with each of two doses of Benadryl, 1 0 and 3.0 mgm /kgm , intravenously, and a similar number of experiments were made with atropine sulfate. Benadryl and atropine were injected at a constant rate during one minute in order to minimize depressor effects which were known to follow rapid injections of Benadryl The dose of atropine chosen for use was 0 03 mgm./kgm. It represents $\frac{1}{30}$ and $\frac{1}{100}$ of the doses of Benadryl employed and was selected on the basis that Benadryl was $\frac{1}{30}$ as effective as atropine in antagonizing the action of acetylcholine on intestinal muscle.

TABLE 3

Effects of benadryl and atropine on alterations in blood pressure following injections of epinephrine, histamine and acetylcholine

DEPRESSOR AND PRESSOR AGENTS*	DOSE RANGE	NO. OF INJECTIONS	RESPONSES BEFORE		RESPONSES AFTER		$M_1 - M_2$ ± P.E	DIFFERENCE BETWEEN MEANS
			Range	Mean ± P.E.	Range	Mean ± P.E.		
A. Benadryl, 1.0 mgm./kgm.; 4 dogs								
	γ		*mm Hg*		*mm Hg*			*per cent*
Histamine diphosphate .	7-15	12	−12-−30	−21.1 ±1 15	−4-−14	−6 58 ±0 67	−14 5 ±1 33	−68
Acetylcholine bromide .	3-15	12	−10-−35	−21 9 ±1.62	0-−25	−13 1 ±1.46	−8 8 ±2 18	−40
Epinephrine hydrochloride	10	12	+32-+70	+49.2 ±1 88	+28-+66	+48.0 ±2.73	−1 2 ±3.31	−2 4
B. Benadryl, 3.0 mgm./kgm.; 4 dogs								
Histamine diphosphate ..	4-10	12	−10-−30	−16.7 ±1 16	−2-−13	−7.0 ±0 64	−9.7 ±1 32	−58
Acetylcholine bromide .	5-50	12	−12-−29	−17 5 ±1 03	0-−13	−6 5 ±0.87	−11.0 ±1 34	−63
Epinephrine hydrochloride	10	10	+21-+60	+45 ±2 96	+37-+90	+64 ±3.89	+19.0 ±4 88	+42
C. Atropine sulfate, 0.03 mgm./kgm ; 4 dogs								
Histamine diphosphate ..	4-15	12	−12-−23	−17.8 ±0 81	−8-−23	−15 4 ±0.95	−2.4 ±1.24	−13.4
Acetylcholine bromide .	2-60	12	−11-−22	−16 7 ±0.66	0-−4	−0 5 ±0 21	−16 2 ±0 69	−97
Epinephrine hydrochloride	10	12	+12-+60	+42.6 ±3 22	+23-+54	+60.5 ±4 49	+17.9 ±5 52	+42

* Three injections of histamine, acetylcholine and epinephrine made intravenously in each of four dogs before and after intravenous injection of (A) benadryl, 1 0 mgm /kgm , (B) benadryl, 3 0 mgm /kgm , and (C) atropine sulfate 0 03 mgm /kgm.

The data in table 3 (Part A) reveal that depressor responses of 12 to 30 mm. Hg following small doses of histamine were reduced to 4 to 14 mm. Hg following administration of Benadryl (1.0 mgm./kgm., I.V.). The mean depressor response to histamine was reduced 68 per cent. Likewise, the depressor responses to small doses of acetylcholine were significantly reduced, the mean response being decreased 40 per cent. On the other hand, the mean pressor response to epinephrine was not significantly altered in either direction.

Repetition of these experiments in another four dogs with the dose of Benadryl increased to 3.0 mgm./kgm., intravenously (table 3, Part B), revealed that the mean depressor responses to histamine and acetylcholine were reduced 58 and 63 per cent, respectively. Thus, an increase in the dose of Benadryl failed to obliterate the depressor effects of histamine and acetylcholine. The inhibitory effect of the increased dose of Benadryl against histamine was no greater than that which obtained with the dose of 1.0 mgm./kgm. Acetylcholine was antagonized to a greater degree. A result not evident in previous experiments was that the mean pressor response to epinephrine was increased 42 per cent (table 3 and figure 1).

An additional group of experiments was made on four dogs in which atropine sulfate (0.03 mgm./kgm., I.V.) was substituted for Benadryl (table 3, Part C). These experiments were made to serve as controls, in some degree, for the experiments with Benadryl and also to aid in interpreting the previous findings. The dose of atropine used failed to significantly alter the depressor response to to histamine but the antagonism of acetylcholine was nearly complete in each experiment. Furthermore, the mean pressor response to epinephrine was augmented 42 per cent. The accentuated response is equal in magnitude to that obtaining after Benadryl at a dose of 3.0 mgm./kgm. and suggests that this effect of Benadryl is referable to a weak atropine-like activity, although other causes must be considered.

Other facts pertaining to the pharmacological actions of Benadryl were ascertained during the course of these experiments. Following the intravenous administration of the relative small doses of drug employed, the antagonism of the vascular effects of histamine and acetylcholine persisted for 1½ to 2 hours. When the augmented pressor response to epinephrine became evident it endured for a similar period of time. It is apparent that the activity of Benadryl persists for an appreciable time interval.

Effects of Benadryl on blood pressure and respiration as observed in these experiments are of interest. Preliminary studies had revealed that rapid intravenous injections elicited hypotension and apnea, both of short duration. It was because of this observation that in subsequent experiments even small doses of the drug were *injected at a constant rate during one minute.* Doses of 1.0 mgm./kgm. reduced blood pressure levels of 100 to 150 mm. Hg by 5 to 10 mm. Hg in three of the four experiments. One minute following injection of the drug the blood pressure had returned to the control level and then immediately was elevated 5 to 12 mm. Hg. These slight pressor responses persisted for only 3 to 8 minutes. When the dose of Benadryl was increased to 3.0 mgm./kgm. the diphasic alterations in blood pressure were similar but of greater magnitude. Depressor responses recorded in three of the four experiments amounted to 10 to 30 mm. Hg and persisted for less than two minutes. Pressor responses of 5 to 28 mm. Hg appeared consistently; the blood pressure progressively decreased to normal within 12 to 25 minutes. It was not possible to determine the cause of the pressor or depressor effects from records obtained under the experimental conditions employed. Neither the pressor nor depressor responses were of sufficient degree or duration to suggest that they were intimately related to the prolonged action of Benadryl in antagonizing the depressor action of histamine and acetylcholine or in potentiating the pressor action of epinephrine.

Respiration was unaffected by Benadryl when doses of 1.0 mgm./kgm. were injected intravenously. With the larger dose of 3.0 mgm./kgm., the depth of respiration was slightly decreased for one minute. Respiratory rates were increased 20 to 40 per cent, usually for only 1 to 3 minutes, but in one experiment a slightly increased rate persisted for 20 minutes.

DISCUSSION. Previous studies with guinea pigs (1, 2) indicate that Benadryl is markedly effective in preventing mortality due to severe bronchoconstriction. In other experiments (11) it was demonstrated that Benadryl dilates the bronchioles of isolated, perfused lungs and also relaxes the bronchioles after spasm has been induced with histamine. This drug therefore possesses a marked bronchodilator activity and a singular capacity to antagonize histamine.

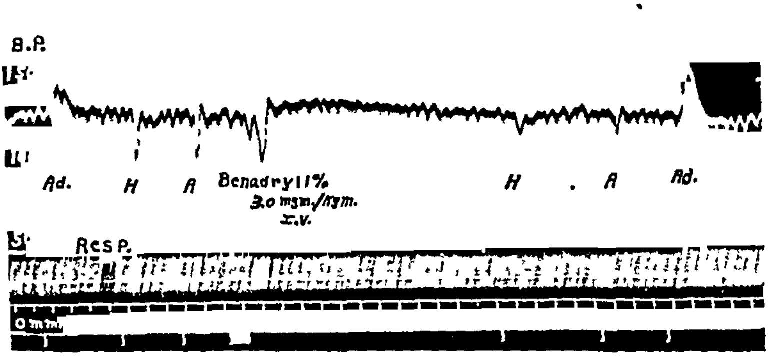

FIG. 1. EFFECT OF BENADRYL ON ALTERATIONS IN BLOOD PRESSURE OF PHENOBARBITALIZED DOG FOLLOWING INJECTIONS OF EPINEPHRINE, HISTAMINE AND ACETYLCHOLINE

Records from above downwards: blood pressure, respiration, time in 1 min. intervals; 0 mm. Hg baseline and signal marker.

Pressor and depressor responses before and after administration of benadryl, 3.0 mgm./kgm., intravenously. Ad = epinephrine hydrochloride, 10γ intravenously, H = histamine diphosphate, 10γ intravenously, A = acetylcholine bromide, 10γ intravenously.

In the present studies with isolated guinea pig ileum it was revealed that Benadryl, in a dilution of 1:50,000,000, was capable of preventing histamine-induced spasm, whereas dilutions of 1:4,000,000 and 1:125,000 were required to antagonize acetylcholine and barium respectively. The drug is therefore an exceedingly potent, specific inhibitor of histamine in the same sense that atropine is regarded as a specific inhibitor of acetylcholine. Atropine and Benadryl are examples of antispasmodic agents with singular properties of antagonizing acetylcholine and histamine respectively.

The term musculotropic is frequently used when referring to smooth muscle stimulants such as histamine and barium or to antispasmodic agents which antagonize such stimulants. Both Benadryl and 1571F (table 1) are potent histamine antagonists but less potent as barium antagonists than Trasentin and Pavatrine (19). In view of the specific action of compounds such as Benadryl we prefer to employ the descriptive term anti-histamine compound or histamine antagonist and use musculotropic as a generic term to include all agents acting directly on muscle cells.

The demonstration of compounds with specific anti-histamine action emphasizes the importance of including histamine as a spasmogenic agent when evaluating synthetic antispasmodics. The rather common practise of testing antispasmodics only for their ability to antagonize barium and acetylcholine obviously would not permit detection of anti-histamine drugs. Such drugs might prove useful as research tools and therapeutic agents, especially in view of the presence and wide distribution of histamine in animal tissues and the evidence regarding its rôle in physiological and pathological mechanisms.

Benadryl also prevented the relaxing effect of histamine on the vascular system as indicated by the fact that administration of the drug to dogs decreased the depressor responses to small intravenous doses of histamine. These findings are in agreement with results published by Wells *et al.* (12, 13). Depressor responses following small doses of acetylcholine were also partially prevented. Its effectiveness in antagonizing acetylcholine is approximately $\frac{1}{50}$ that of atropine, as indicated by results of studies on blood pressure as well as on intestinal muscle.

The weak atropine-like activity exerted by Benadryl may have accounted for the augmentation of pressor response to epinephrine. However, other explanations are possible since Benadryl itself produced measurable pressor effects and could have sensitized the heart or vascular system so as to increase the effectiveness of epinephrine. The fleeting hypotension following injections of Benadryl and the slight pressor effect of short duration are probably not closely related to the ability of the drug to diminish the depressor effects of histamine and acetylcholine for 1 or 2 hours, or longer. Furthermore, the maintenance of blood pressure at or near normal levels suggests that the phenomena demonstrated are due to specific actions of Benadryl and not to general vascular depression.

Although several synthetic compounds which antagonize histamine are capable of producing epinephrine reversal (14–18), Benadryl does not possess this property.

Gastric secretion induced by administration of histamine to dogs with denervated gastric pouches was significantly reduced by injecting Benadryl subcutaneously. However, the secretogogue action of histamine was not inhibited in one of four dogs. It does not appear logical to conclude that Benadryl definitely antagonized the secretogogue action of histamine in view of the dose administered, the marked potency demonstrated in other types of experiments, and the moderate degree of gastric secretory inhibition which was not produced consistently. The degree of inhibition demonstrated might be referable to changes in motility, vascularity, or other alteration in function and not to any direct effect on gastric secretory cells which involved the antagonism of histamine. Only the effect of Benadryl on histamine, a humoral stimulus of the gastric secretory cells, was studied. Since a weak atropine-like action has been demonstrated, Benadryl might be more effective in suppressing gastric secretion of the intact, innervated stomach when both humoral and nervous stimuli were operating.

The experimental study supplies some information concerning the possible mode of action of Benadryl. It is doubtful whether it inactivates histamine because of chemical union or neutralization for if so one would expect greater antagonism of the secretogogue action of histamine. Furthermore, Benadryl in a dilution of 1:50,000,000 was effective in preventing the spasmogenic action of histamine diluted 1:12,500,000; obviously, stoichiometric neutralization does not explain the antagonism. Furthermore, low dilutions of Benadryl prevented the spasmogenic action of barium and acetylcholine on intestinal smooth muscle. It is unlikely that Benadryl chemically neutralizes such dissimilar substances as histamine, barium and acetylcholine; the antagonism of all three suggests the more probable explanation that the drug alters permeability of smooth muscle or combines with some portion of the effector cell so as to prevent histamine, or other spasmogenic agents, from producing a normal response. Gastric secretory cells may be less susceptible to changes in permeability or such cells may not contain elements which readily unite with Benadryl and thereby exclude histamine from combining or acting. Wells *et al.* (12, 13) concluded from quantitative studies of Benadryl-histamine antagonism relative to blood pressure responses in dogs that "...antagonism of histamine by Benadryl may well be due to adsorption of Benadryl onto the effector mechanism, competing thereby, with histamine for its site of action."

SUMMARY

Pharmacological studies with a synthetic compound, Benadryl (β-dimethylaminoethyl benzhydryl ether hydrochloride), revealed the following:

1. A dilution of 1:50,000,000 proved effective in antagonizing the spasmogenic effects of histamine on intestinal muscle whereas much larger doses were required to antagonize the effects of acetylcholine and barium. Benadryl possesses a degree of specificity against histamine which approaches the specificity of atropine against acetylcholine. In view of weak antagonism of barium, the term anti-histamine compound describes Benadryl more specifically than use of the generic term, musculotropic antispasmodic.
2. Secretion from denervated gastric pouches in dogs following stimulation with histamine was reduced approximately 40 per cent by pre-treatment with Benadryl. Evidence of inconsistency in results and lack of a pronounced inhibitory effect reduces the probability that a direct antagonism of the secretogogue action of histamine was involved.
3. Depressor effects of small doses of histamine and acetylcholine were decreased by intravenous administration of Benadryl in dogs. The possibility exists, therefore, that the relaxing effect of histamine on vascular smooth muscle was antagonized, as well as the contracting effect as demonstrated on intestinal muscle.
4. Adequate doses of Benadryl augmented the pressor response to epinephrine. There was no evidence of epinephrine reversal which has been demonstrated with several other synthetic drugs which possess some degree of anti-histamine action.

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STUDIES ON THE FATE OF NICOTINE IN THE BODY

IV. OBSERVATIONS ON THE CHEMICAL STRUCTURE OF AN END PRODUCT OF NICOTINE METABOLISM

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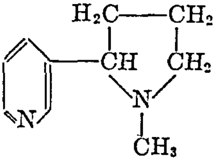
We have previously shown for both man (1) and the dog (2) that only about 10 per cent of administered nicotine is excreted unchanged in the urine. The fate of the remaining 90 per cent remains to be elucidated.

Believing that the pyrrolidine component of the nicotine molecule was the most probable site of chemical change, the possibility that this component was either completely split off or reduced to a one carbon side chain was studied (2). Failure to detect any increase in the excretion of N-methyl pyridinium hydroxide, nicotinic acid, nicotinuric acid or trigonelline following nicotine administration largely negated this possibility. However, during the course of these studies it was found that the urine of dogs that had received nicotine contained a substance that gave a red color when reacted with cyanogen bromide (CNBr). This substance was absent from the urine of dogs that had not received nicotine and did not represent unchanged nicotine since nicotine yields a greenish yellow color with CNBr.

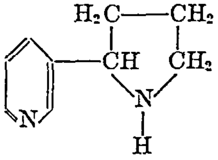
With this clue in mind, the possibility that the nicotine molecule is methylated or demethylated in the course of its metabolism was examined (3) by testing the urine of dogs for the presence of a substance yielding a red color with cyanogen bromide following administration of monomethyl nicotinium iodide, isomonomethyl nicotinium iodide, dimethyl nicotinium di-iodide and nornicotine. Positive results were obtained only in the case of nornicotine. It was further found that nornicotine itself yields a red color when reacted with CNBr in the presence of phosphate ion raising the possibility that nornicotine is an end product of nicotine metabolism. This, however, was ruled out by the fact that the nicotine metabolite yielding a red color with CNBr, unlike nornicotine, is not extractable from alkalinized urine with ether. Nor does nornicotine metabolism proceed in the same manner as nicotine metabolism, for unchanged nornicotine was found to be entirely responsible for the red color formed by reacting urine obtained following nornicotine administration with CNBr.

These experiments, however, gave the first clue to the nature of the chemical structure determining the evolution of a red color in the presence of CNBr, for the sole structural difference between nicotine and nornicotine lies in the number of substitutions on the pyrrolidine nitrogen. Since we have seen from the above that demethylation of the nicotine molecule, if it occurs, cannot be the sole change, the next most likely proposition seemed to be that the pyrrolidine ring is split during the course of nicotine metabolism. Accordingly the following studies have been made.

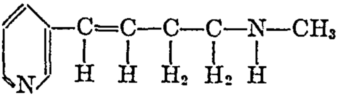
When the pyrrolidine ring of nicotine is split by such chemical procedures as benzoylation the cleavage occurs between the nitrogen and the carbon atom attached to the pyridine ring. Debenzoylation then results in the formation of 3-(4-methylamino-1-butenyl)-pyridine[1](metanicotine). Reacted with CNBr, either in the presence or absence of phosphate ion, this substance does not yield a red color, nor does its hydrogenated derivative 3-(4-methylaminobutyl)-pyridine.[1] Neither does the demethylated analogue 3-(4-aminobutyl)-pyridine[2] yield a red color with CNBr.



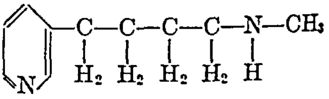
Nicotine



Nornicotine



3-(4-methylamino-1-butenyl)-pyridine



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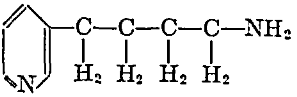
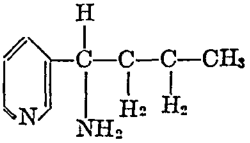


3-(4-aminobutyl)-pyridine



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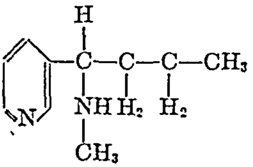

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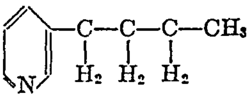

3-butyl-pyridine

[1] Kindly prepared for us by C. H. Rayburn and H. N. Wingfield of the Research Laboratory of the American Tobacco Company.

[2] The preparation of this compound has recently been described by P. G. Haines, A. Eisner and C. F. Woodward (J. A. C. S., 67: 1258, 1945) of the Eastern Regional Research Laboratory of the U. S. Department of Agriculture, who kindly furnished us with a sample for this study.

In view of these findings, the alternate possibility that cleavage of the pyrrolidine ring between the nitrogen and the 5 position occurs during nicotine metabolism, was investigated. To this end the compounds 3-(1-aminobutyl) pyridine[1] and 3-(1-methylaminobutyl)-pyridine[1] were prepared. Reacted with CNBr both yielded a red color. In contrast to this the deaminated analogue 3-butyl-pyridine[1] failed to yield a red color with CNBr.

Interpreting this in the light of our findings concerning the fate of nicotine in the animal body, it seems fair to assume that the nicotine metabolite that yields a red color with CNBr is a product of cleavage of the pyrrolidine ring between the nitrogen and the 5 position. The additional finding that this substance is not extractable with ether from alkalinized urine is suggestive of the presence of a carboxyl ending on the resulting side chain. Whether or not this substance accounts for all or only part of the 90 per cent of administered nicotine that fails to be excreted unchanged, is as yet unknown.

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EFFECTS OF MORPHINE AND ITS DERIVATIVES ON INTERMEDIARY METABOLISM[1]

IV. The Influence of Chronic Morphine and Heroin Poisoning on the Oxygen Consumption of Dog, Rat and Mouse Skeletal Muscle

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It has been demonstrated previously (1) that skeletal muscle of chronically morphinized rats, taken during the first few days of the withdrawal period, has a significantly higher oxygen uptake (as measured in vitro in the Warburg apparatus) than muscle taken from normal animals. Morphine sulfate, added in vitro to minced skeletal muscle from the chronically morphinized as well as the normal rat, produces an increase in oxygen uptake by this tissue. The significance of these findings in relation to addiction could be more firmly established if muscle from rats chronically poisoned with some morphine derivative of known addiction potentialities, or muscle from other animal species poisoned with morphine could be shown to have a higher oxygen consumption than normal muscle.

A preliminary report (2) indicated that skeletal muscle obtained by biopsy from dogs chronically poisoned with morphine had a higher oxygen uptake taken after 48 or 72 hours withdrawal than normal muscle from the same animal before the administration of the drug. It is the purpose of this study to determine whether skeletal muscle from dogs and mice chronically poisoned with morphine, exhibits an increase in oxygen utilization during the withdrawal period (as does muscle from chronically morphinized rats) and whether the same qualitative change in the oxygen metabolism of rat skeletal muscle occurs after prolonged administration of heroin.

Method. Skeletal muscle samples from the dog and mouse were minced in a micromincer (3) and those from the rat were prepared by mincing in a Latapie mincer. After the tissue was minced, 200 mgm. was suspended in 2.0 cc. of Ringer-M/60 phosphate buffer in Warburg flasks. After 10 minutes equilibration at 38°C., oxygen uptake was measured over a period of 1 hour (rat and mouse) or 3 hours (dog). When dog or mouse skeletal muscle were used, 0.12 per cent morphine sulfate was present in half the flasks.

Dog. Specimens of muscle were obtained by sterile biopsy from the biceps femoris of normal dogs anesthetized with intravenous pentobarbital or procaine block of the sciatic nerve. The two methods of anesthesia were employed to determine if the type of drug used influenced the oxygen consumption. After these preliminary studies the daily administration of morphine sulfate (5 mgm. per kgm.) was begun and increased as tolerated, until a dose, as indicated in table 1, was reached. Oxygen uptake values were obtained at the 48 hour withdrawal period on muscle from the same leg as used previously for the determination of normal oxygen consumption.

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[1] This work was begun in the Department of Pharmacology, University of Wisconsin, and supported by the Wisconsin Alumni Research Foundation.

Mouse. A group of 18 normal mice of the same age and approximately the same weight were divided into two equal groups. One group was placed on an adequate mouse diet and the other group was given the same diet to which morphine sulfate had been added in sufficient amount to provide a calculated intake of approximately 20 mgm. per kgm. per day. At the end of one week the food consumption of this group was determined and the actual daily intake of drug calculated. The concentration of morphine in the food was increased at weekly intervals until the intake, at the end of 10 weeks, was 195 mgm. per kgm. per day. At this time the animals were placed on the standard diet containing no drug and 48 hours later killed by decapitation and the muscle removed from both hind legs. The muscle from the animals receiving the morphine-free diet was treated in the same manner.

Rat. Albino rats weighing approximately 200 grams, chosen without regard to sex, were used in these studies. The animals were maintained on an adequate diet. Heroin hydrochloride was administered daily by subcutaneous injection in steadily increasing doses over a period of 52 days. The dose of heroin was raised from 10 to 100 mgm. per kgm. per day during this time. The latter dose was maintained for one week and administration of the drug discontinued. Forty-eight hours after the last dose of the drug, the animals were killed by decapitation and the entire muscle from the right hind leg removed. This was freed of fat, nerve and connective tissue and its oxygen consumption determined. Muscle from normal control animals was treated in a similar manner.

TABLE 1

Oxygen consumption of dog skeletal muscle mince before and after chronic morphine poisoning (48 hours withdrawal)

DOG	DOSE AT TIME OF WITHDRAWAL	TYPE OF ANESTHESIA	Q_{O_2}	
			Control	After morphine
	mgm. per kgm.			
1a	70	I.V. pentobarbital	5.43	7.79
1b	200	I.V. pentobarbital	5.25	7.15
2	70	I.V. pentobarbital procaine	5.49	6.66
3	100	sciatic block	4.67	5.21
5	200	I.V. pentobarbital	5.73	5.85
6	100	I.V. pentobarbital	3.40	4.11
9	100	I.V. pentobarbital	4.49	6.73

Results. The rate of oxygen consumption by minced skeletal muscle of the dog is significantly increased by chronic morphine poisoning if this is determined 48 hours after the last dose of the drug. These data are presented in columns 4 and 5 of table 1. In each case the Q_{O_2} of minced muscle after chronic morphine poisoning is higher than the Q_{O_2} of minced muscle obtained from the same muscle of the same animal before administration of the drug. The mean difference in Q_{O_2} for the 7 animals is 1.29 with a $\sigma = 0.886$ and an S.E.$_{M}$ = 0.325. The t value of the difference is 4.0 which gives a significant value for P lying beyond 0.01.

The type of anesthesia apparently did not have any marked effect on oxygen consumption of the muscle since the Q_{O_2} of muscle when obtained by procaine block of the sciatic nerve lies in the range of values obtained when the animal was anesthetized with intravenous pentobarbital (columns 3 and 4).

There also appears to be no quantitative relationship between dose of drug at time of withdrawal and increased oxygen consumption of muscle.

The mean no-substrate Q_{O_2} for minced skeletal muscle from 9 normal mice was found to be 1.07 with an S.E.$_M$ equal to 0.046 and a t value of M equal to 23. These Q_{O_2} values are presented graphically in the first column of figure 1. In this figure similar data obtained on muscle from chronically morphinized mice are presented for comparison. The mean Q_{O_2} for minced skeletal muscle from this second group of animals is 1.46 with an S.E.$_M$ equal to 0.12 and at value of M equal to 12. The t value of the difference of these two means gives a significant figure of 3.05.

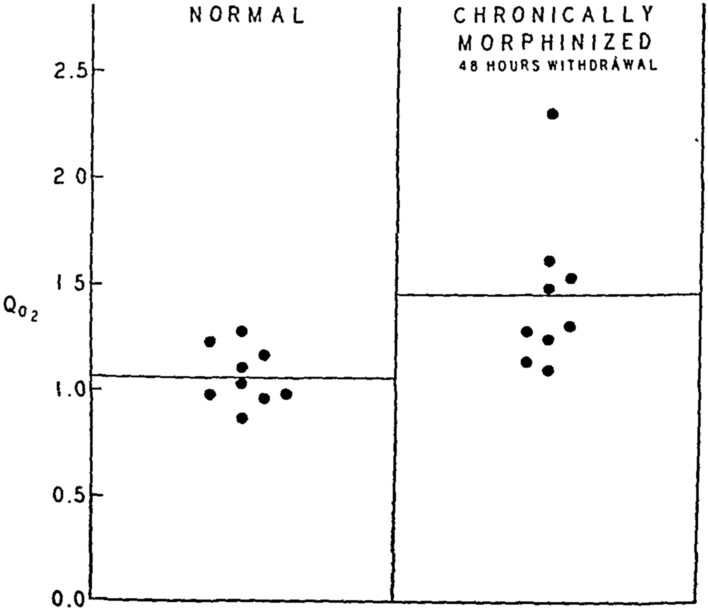

FIG. 1. OXYGEN CONSUMPTION OF MINCED SKELETAL MUSCLE OF NORMAL MICE AND CHRONICALLY MORPHINIZED MICE

Each filled circle represents Q_{O_2} on muscle from one animal. The horizontal line indicates the mean for each group.

Morphine sulfate (0.12 per cent) produces the same qualitative effect when added in vitro to a nomal dog skeletal muscle mince as it does when aded to normal rat skeletal muscle mince (table 2). There is a small, but significant, increase in the Q_{O_2} produced by the addition of the morphine. The U_{O_2} (3 hr.) shows a much greater increase. In contrast to the findings for the rat, addition of morphine to muscle from chronically morphinized dogs has no uniform or significant effect on Q_{O_2} or U_{O_2} (3 hr.).

The oxygen consumption of muscle from normal mice as well as from chronically morphinized mice is not significantly affected by addition of morphine in vitro (table 2).

Skeletal muscle from rats poisoned with heroin behaves, in regard to oxygen consumption, in a manner qualitatively similar to skeletal muscle from chronically morphinized rats. The mean Q_{O_2} of minced skeletal muscle from 19 normal rats was found to be 2.04 (first column of Figure 2) with a t value of 17.3. Muscle taken from 5 rats chronically poisoned with heroin, 48 hours after the last dose of the drug, had a mean Q_{O_2} of 3.16 (t value of mean = 14.4). This value is significantly higher than the mean for normal muscle (t value of the difference of the means = 4.5).

Discussion. Although the results presented in this paper do not yield any information as to the actual mechanisms involved in the production of an increased oxidative metabolism of skeletal muscle arising as a result of chronic morphine poisoning, they do lend support to the view that this heightened metabolism is in some way related to tolerance, addiction and the signs of withdrawal. If such a finding were to obtain in only one species and with morphine only one would be hesitant in attaching too much importance to it in connection with addiction. However, it occurs not in the rat alone, but also in the mouse and dog and in the rat with heroin as well as with morphine.

TABLE 2

Effect of 0.12 per cent morphine sulfate on the Q_{O_2} and U_{O_2} (3 hr.) of minced skeletal muscle from normal and chronically morphinized dogs and mice

Values are expressed as the mean difference in oxygen uptake between a control and one to which morphine sulfate had been added

ANIMAL		NUMBER OF ANIMALS	MEAN DIFFERENCE IN Q_{O_2}		MEAN DIFFERENCE IN U_{O_2} (3 HR.)	
Dog	Normal	7	+0.22	(t value of difference = 19.6)	+1.34	(t value of difference = 0.91)
	Chronically morphinized	7	−0.60	(t value of difference = 3.34)	+0.49	(t value of difference = 0.91)
Mouse	Normal	9	+0.04			
	Chronically morphinized	9	−0.19	(t value of difference = 1.39)		

As with the rat, the oxygen uptake of skeletal muscle from chronically morphinized dogs was found to be greatest 48 nours after the last dose of the drug. At this time all samples of muscle exhibited a significant increase in oxygen uptake as compared to control samples. In some animals high values were obtained at 24, 72, and 96 hours but this was not uniform and the mean increase of all animals was not significant at these periods. By analogy with the rat and mouse we would expect oxidative metabolism to be significantly increased at 24, 72 and 96 hours even though the peak in all three species comes at 48 hours.

It must be pointed out that in the smaller animals the results represent a mean value for all muscle of the hind quarters, whereas in the dog an attempt was made to sample a single muscle at intervals. It will be noted that while the peak value at 48 hours in the dog is significant it does not represent as great a total increment as in the rat and mouse. It seems possible, therefore, that there could be factors tending to give low values such as sampling errors (excising an adjoining muscle having a lower oxidative metabolism than the biceps femoris), inclusion in the sample of connective tissue from the previous operative site, and local changes in blood and nerve supply as a result of the first surgical procedure, which although operative in all instances might be adequate to mask small but otherwise significant increases at 24, 72 and 96 hours but insufficient to obscure the peak increase at 48 hours.

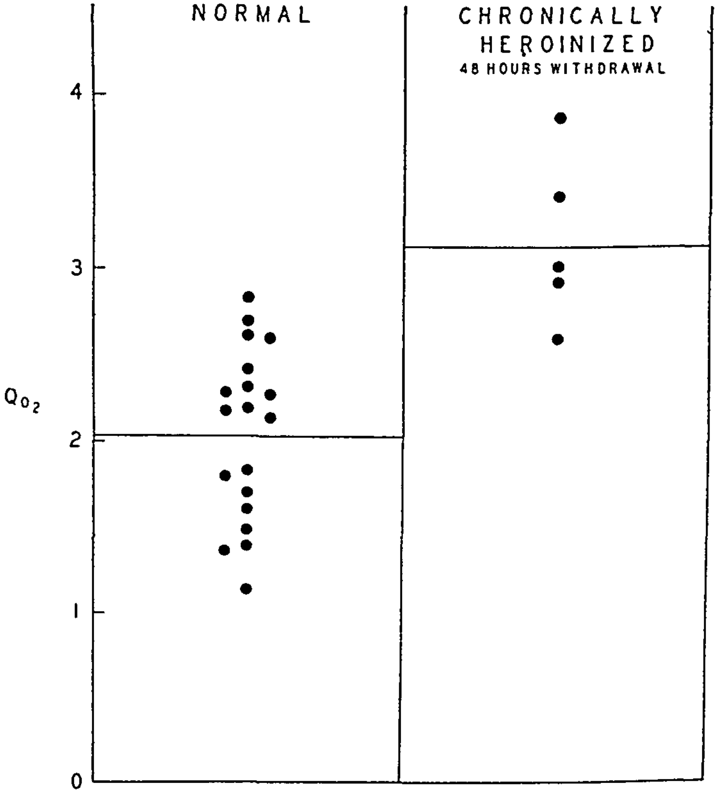

Fig. 2. Oxygen Consumption of Minced Skeletal Muscle of Normal Rats and Rats Chronically Poisoned with Heroin

Each filled circle represents Q_{O_2} on muscle from one animal. The horizontal line indicates the mean for each group.

Although addition of morphine sulfate in vitro to a normal dog skeletal muscle mince produces an increase in oxygen consumption qualitatively similar to that obtained when normal rat skeletal muscle respires in the presence of morphine sulfate, such is not the case with muscle from normal mice. Unlike the rat, addition of morphine sulfate to skeletal muscle from dogs or mice chronically poisoned with morphine produces no significant alteration in oxygen uptake. The mechanism whereby an increase in oxygen utilization is brought about by an addition in vitro of morphine sulfate to rat skeletal muscle is being investigated but has not been elucidated. Until this mechanism is established, the lack of effect on skeletal muscle from the mouse and chronically morphinized dog cannot be postulated.

SUMMARY

Skeletal muscle obtained from chronically morphinized dogs and mice forty-eight hours following withdrawal of the drug has a significantly greater oxygen uptake than muscle taken from normal animals. Chronic poisoning with heroin produces an increase in the oxygen consumption of rat skeletal muscle qualitatively like that produced by chronic morphine poisoning.

Addition of morphine sulfate (0.12 per cent) to a skeletal muscle mince from normal dogs produces a significant increase in the oxygen uptake. Addition of morphine sulfate (0.12 per cent) to a skeletal muscle mince from normal mice or chronically morphinized mice or dogs causes no significant alteration in oxygen consumption.

The author is indebted to Dr. M. H. Seevers for suggestions and criticisms and to Mr. J. K. Theisen for technical assistance.

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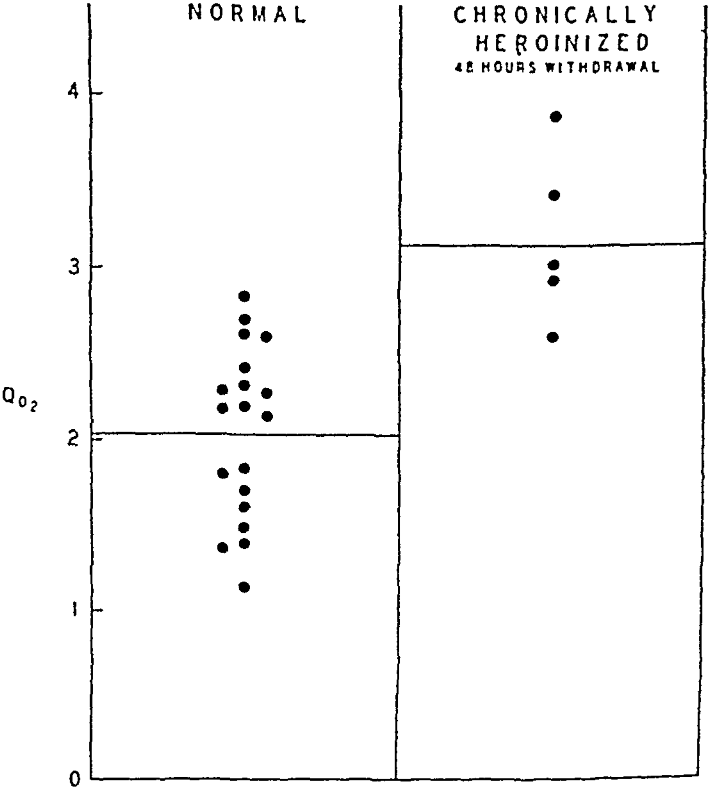

FIG. 2. OXYGEN CONSUMPTION OF MINCED SKELETAL MUSCLE OF NORMAL RATS AND RATS CHRONICALLY POISONED WITH HEROIN

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ON THE ELIMINATION OF G-STROPHANTHIN BY THE RAT

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The rat is known to be one of the most resistant animals to the toxic action of cardioactive glycosides (1, 2, 3, 4, 5, 6). This resistance is partly due to the ability of the rat heart to function in the presence of high concentrations of these glycosides (1, 7). Another cause for this resistance is the ability of the rat to eliminate large quantities of these drugs (5, 6). Straub believes that with k-strophanthin the elimination process is mainly due to the ability of rat blood to destroy this glycoside (2). On the other hand, Hatcher (3) and Hatcher and Eggleston (4) present strong evidence that this process of elimination is due largely to an excretion of the drug via the liver into the gastro-intestinal tract. This is then followed by a partial destruction of the material in the large intestine; the rest is excreted in the feces.

The object of the present study was to determine the relative importance of the liver in this process of elimination.

We have studied the influence of hepatectomy on the lethal dose (L.D.) of g-strophanthin and have demonstrated a reduction of this dose, which is related to the degree of hepatectomy. The process of hypertrophy of the liver following partial hepatectomy has also been correlated with the return to normal values of the lethal dose of g-strophanthin.

In another set of experiments the rate of elimination of g-strophanthin in the normal animal has been compared with that of the "functionally hepatectomised" (eviscerated) rat. Our data show that evisceration markedly reduces the process of elimination.

A number of in vitro experiments have been designed to see whether blood and liver slices of the rat can destroy g-strophanthin.

Materials and methods. The animals employed were white rats, raised locally on a basic diet of barley and seasonal vegetables, supplemented every other day with a special diet containing per kg., 500 grams barley flour, 3–4 eggs, 200 cc. of milk and 200 grams of ground mutton. The rats were in good health and showed no obvious signs of nutritional deficiency. Prior to starting the experiment, all animals were kept for 4–6 hours without food. Adult male rats only were used, since Holck and al. (8) have demonstrated sex and age differences in the sensitivity of rats to g-strophanthin.

The g-strophanthin used was a crystalline material supplied by Burroughs Wellcome (Ouabain B.P.C. 13% water of crystallization). In all experiments a freshly prepared 0.5 per cent solution in normal saline was used.

The lethal dose of g-strophanthin was determined by infusing the solution of g-strophanthin at a constant rate into the external jugular vein of anaesthetised rats (0.06–0.08 gram amytal per kg. body weight, given intraperitoneally). Since in the rat respiratory paralysis may occasionally precede cardiac arrest, we have employed artificial respiration in all our experiments.

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To facilitate the observation of the end point of the experiments, namely, ventricular fibrillation or cardiac arrest, the chest was opened by means of a midline incision and the pericardium removed. After careful haemostasis, 15-20 units of heparin were given intravenously so as to prevent clotting of the blood in the venous cannula employed for the infusion. Mehnert (6) has shown that heparin in the amounts used does not modify the lethal dose of k-strophanthin.

In the rat the lethal dose of g-strophanthin is largely influenced by the rate of infusion. We have chosen a rate of about 54 mg. per kg. per hour, since with this rate cardiac arrest in the rat occurs within a period of 60 to 100 mniutes. Constant infusions were made by means of a locally constructed apparatus consisting of an electric motor, gears and a rack and pinion which pushed the piston of a tuberculin syringe. By changing the gears and syringes the apparatus could be adjusted to deliver at rates varying between 0.004 and 0.184 cc. per minute with an error not exceeding ±5 per cent.

Partial hepatectomy was performed by removing various portions of the liver. Thus approximately 8 per cent hepatectomy was made by removing the smaller portion of the median lobe, and approximately 20 per cent by removing the larger part of the same lobe. Total extirpation of this corresponds to about 30 per cent hepatectomy, while the removal of both median and left lateral lobes is equivalent to about 67 per cent.

Total hepatectomy was performed by evisceration, a procedure shown by Russel (9) to give "functionally" hepatectomised rats. Following partial or complete (functional) hepatectomy the lethal dose of g-strophanthin was determined using the same rate of administration of about 54 mg. per kg. per hour, the rate of administration being based on the body weight, minus the weight of the organs extirpated. In all these experiments wet liver weights were recorded.

The experiments on liver regeneration were performed on animals where both the median and left lateral lobes (67% of the liver) had been removed. The general procedure was the same as that of Higgins and Anderson (10). The lethal dose of g-strophanthin was determined after 2, 4, 7, 14 and 21 days. In 32 normal animals the average liver weight was found to be 3.31, ±0.46 per cent, of the body weight. This value is lower than those of both Hata (11), and Higgins and Anderson (10). The percentage hypertrophy of the liver was calculated from the liver and body weights, one hundred per cent hypertrophy being that liver weight which was equal to 3.31 per cent of the body weight.

The rate of elimination of g-strophanthin was determined by a method developed by Hauptstein (12) and Heubner and Nyary (5). This consisted essentially of the determination of the lethal dose of the glycoside at different rates of administration. Lowering of the rate of administration resulted in an increase in the lethal dose. By dividing the differences between these lethal doses by the differences in the corresponding experimental times, the rate of elimination per kg. per hour could be determined.

The in vitro experiments with rat blood, designed to see whether this tissue is able to destroy g-strophanthin, were performed on defibrinated blood diluted 1:1 with Krebs-Henseleit solution (13). After the addition of 1.0 or 1.5 mg. of g-strophanthin to 10 or 15 cc. of this diluted blood it was incubated in a water bath at 38°C. Oxygen was passed through in a continuous stream. Immediately after the addition of g-strophanthin, and again 5 and 9 hours later, 1.5 cc. of the solution was removed, diluted to 22.5 cc. with Krebs solution for cold-blooded animals and tested on a number of isolated frog hearts. Control experiments with diluted rat blood containing no g-strophanthin showed no toxic effects upon the frog hearts.

In the in vitro experiments with rat liver slices about 3.5 gram of slices were placed in 10 or 15 cc. of 1:10,000 solution of g-strophanthin in Krebs solution. This was incubated and oxygenated as described above. Immediately after the liver slices were placed in the solution, and at various time intervals therafter, 1.5 cc. samples of the supernatant fluid were removed, diluted to 22.5 cc. with Krebs solution and tested on a number of frog hearts.

The extraction of g-strophanthin from liver slices was made by grinding the tissue with sand and extracting the brei with 5 cc. of 66 per cent ethyl alcohol for every one gram of liver. This extract was boiled for 2-3 minutes in a water bath, allowed to cool, the original volume restored by adding alcohol, and then centrifuged. An aliquot portion of this alcoholic extract was evaporated on a water bath to about one tenth its volume. The residue was taken up in Krebs solution to give a 1:2 dilution for every one cc. of alcoholic extract evaporated. This extract was filtered and then tested on isolated frog hearts. Control experiments with liver slices where no g-strophanthin was added were non-toxic. Furthermore, g-strophanthin added to these extracts gave the usual response of the frog heart. G-strophanthin added to rat liver brei could be recovered quantitatively within the limit of accuracy of the biological method of assay.

The collection of rat bile was made by cannulating the common bile duct of the anaesthetised rat, collecting the bile and then testing it on the isolated frog heart in a dilution of 1:125. It could be shown that normal rat bile in such concentration does not produce any toxic manifestations in the frog heart. After collecting normal bile for 30–60 minutes, g-strophanthin was infused intravenously at a rate of 25 to 32 mg. per kg. per hour. At one hourly intervals the bile was collected, diluted 1:125 with Locke solution and tested on isolated frog hearts. By comparing the effects on the frog heart produced by bile with known concentrations of g-strophanthin it was possible to estimate the quantity of g-strophanthin eliminated with the bile.

Results. At the rate of administration of approximately 54 mg. per kg. per hour of g-strophanthin cardiac arrest usually occurs within 60 to 100 minutes. The average lethal dose of 10 determinations in anaesthetised male rats was 77.11 ± 7.91 mg. per kg.

The removal of 67 per cent of the liver or total (functional) hepatectomy reduces the lethal dose of g-strophanthin to 13.65 and 9.44 mg. per kg. body weight respectively. The question arises whether the shock produced by partial hepatectomy or evisceration has any influence on the lethal dose. In 6 animals where the abdomen of the rats was opened, the liver pulled out and then replaced, the lethal dose of g-strophanthin was found to be 82.14 ± 8.54 mg. per kg. when the rate of infusion was 53.94 mg. per kg. per hour. In 4 other rats both the median and left lateral lobes were removed under ether anaesthesia. Four to six hours following this operation the animals had apparently well recovered. They were then anaesthetised with amytal and the lethal dose was not significantly different from that determined without allowing the animals to recover from hepatectomy. On the basis of these findings it is justifiable to assume that the lowering of the lethal dose following hepatectomy was due to the removal of the liver and not to the operative procedure and shock. The importance of the gastro-intestinal tract in the determination of the lethal dose of g-strophanthin can be decided by a comparison of the data on rats where evisceration *and* 67 per cent hepatectomy was performed (see table 1). A removal of 67.2 per cent of the liver reduced the lethal dose by 82.3 per cent while evisceration reduced it by 87.8 per cent. From this it must be concluded that the main reduction in the lethal dose by the process of evisceration is due to removal of the liver.

The relationship of percentage hepatectomy to the lethal dose of g-strophanthin was studied on 49 rats of which 39 had been hepatectomised to a variable extent (table 1). This relationship is not a straight line if plotted on a linear scale. However, by plotting the lethal dose against the log of percentage hepatectomy between 8.5 and 100 per cent a straight line is obtained (figure 1).

Hypertrophy of the liver in the rat following partial hepatectomy is a very rapid process (10). We have correlated the changes in the lethal dose of g-strophanthin with the progress of liver hypertrophy and our data on 44 rats are presented in table 2. *With the increase in liver weight the lethal dose of g-strophanthin increases.* From figure 2a it can be seen that liver hypertrophy does not run parallel to the increase in the lethal dose of g-strophanthin. It has been pointed out that the relationship of percentage hepatectomy and lethal dose of g-strophanthin is not a straight line. In order to see whether the discrepancy between liver hypertrophy and increase in the lethal dose is a real one we have plotted in figure 2b liver weight against lethal dose. It can be seen that the discrepancy between liver hypertrophy and lethal dose of g-strophanthin is still apparent.

The data concerning the influence of evisceration on the elimination of g-strophanthin by the rat were obtained on 50 animals and are given in tables 3 and 4.

TABLE 1

The effect of acute hepatectomy on the lethal dose of g-strophanthin in the rat

NUMBER OF RATS	WEIGHT IN *GRAMS*			RATE OF ADMINISTRATION OF g-STROPHANTHIN				LETHAL DOSE OF *g-STROPHANTHIN*				EXPERIMENTAL TIME IN *MINUTES*				PERCENTAGE HEPATECTOMY			
	Average	Maximum	Minimum	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error
				mg. per kg. per hour				*mg. per kg.*											
10	209	256	154	54.43	57.70	51.63	0.65	77.17	107.90	37.50	7.94	85.4	121	39	9.16	Normal			
7	189	212	143	54.87	57.72	51.73	0.88	71.68	98.25	31.91	7.62	78	104	37	8.94	8.5	10.1	7.3	0.43
9	181	220	151	54.63	57.15	53.33	0.44	50.98	78.12	23.17	4.15	56	82	26	6.75	22.1	26.5	19.8	0.75
8	191	268	149	54.20	55.87	51.90	0.40	40.25	63.12	31.55	3.77	44.6	70	34	4.13	31.01	32.4	30.0	0.29
8	217	260	148	53.93	56.22	51.72	0.52	13.65	19.48	4.43	1.54	15.1	21	5.5	1.57	67.2	70.9	63	0.57
7	221	257	166	54.10	57.08	51.84	0.66	9.44	11.83	7.61	0.46	10.6	13	8	0.77	100	(Eviscerated)		

It can be seen that evisceration markedly reduces the process of elimination. If the blood were the most important organ in this elimination process, evisceration should not have affected it to such an extent. The slight elimination seen in eviscerated rats may be due to destruction of g-strophanthin by blood or some other tissue, or due to an excretion process in the kidneys. Hatcher and Eggleston (4) showed conclusively that appreciable quantities of g-strophanthin appear in the urine, especially if the bile duct is tied. It is possible that at least a part of the elimination process still operative in the eviscerated rat is excretion of the glycoside by the kidneys.

Straub (2) claims that the elimination of k-strophanthin in the rat is mainly due to a destruction of this glycoside in the blood. This claim was based on *in vitro* experiments with rat blood and blood extracts. In 4 experiments with diluted rat blood, one of which is given in table 5, we were unable to demonstrate any decrease in the activity of a g-strophanthin solution even after 9 hours of incubation at 38°C.

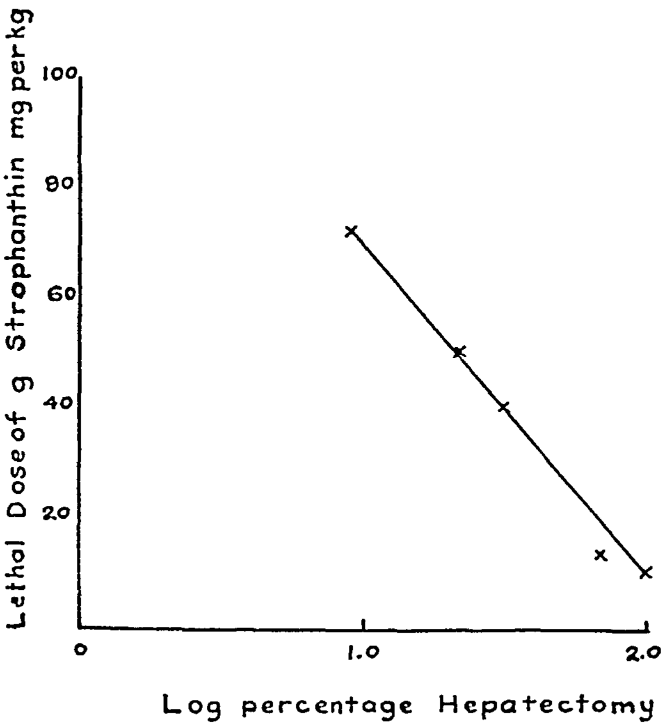


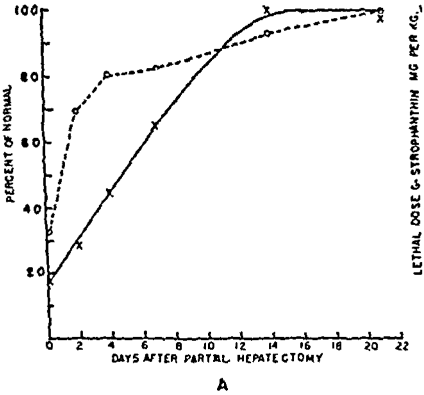
FIG. 1. The relationship of log percentage hepatectomy to the lethal dose of g-strophanthin in the rat.

TABLE 2

The effect of liver regeneration on the lethal dose of g-strophanthin in the rat

NUMBER OF RATS	WEIGHT IN GRAMS			RATE OF ADMINISTRATION OF g-STROPHANTHIN				LETHAL DOSE OF g-STROPHANTHIN				EXPERIMENTAL TIME IN MINUTES				LIVER WEIGHT PER CENT OF NORMAL				DAYS AFTER PARTIAL HEPATECTOMY
	Average	Maximum	Minimum	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	
				mg. per kg. per hour				*mg. per kg.*												
8	217	260	148	53.93	56.22	51.72	0.521	13.65	19.48	4.43	1.537	15.1	21	5.5	1.566	32.8	37.0	29.1	0.572	0
8	178	230	140	53.99	57.38	52.62	0.562	22.09	27.10	12.19	1.795	24.5	31	14	2.04	68.6	80.3	51.2	3.313	2
6	157	185	113	53.86	56.01	52.15	0.674	34.58	48.44	26.62	3.823	36.4	53	30	4.178	80.1	91.3	70.4	4.049	4
8	202	244	153	54.58	56.65	52.12	0.493	50.64	80.86	27.2	6.731	55.9	90	30	7.289	82.6	89.4	67.8	3.542	7
7	174	203	147	54.43	56.74	52.51	0.633	76.76	117.19	40.12	9.064	85.0	132	45	10.162	92.8	105.8	85.5	3.213	14
8	175	200	146	54.79	58.09	52.45	0.707	75.10	104.91	38.07	7.702	82.8	120	40	9.468	99.5	106.5	90.0	4.412	21

Since the liver plays such an important rôle in the fate of g-strophanthin, we investigated whether liver slices can destroy this glycoside. From table 6 it is clear that within 3 hours a reduction of the concentration of g-strophanthin in the supernatant fluid is detectable which does not become greater even after a 9 hour incubation. This reduction, which in the experiment of table 6 was from 1:10,000 to about 1:25,000, might have been due to a destruction of g-strophanthin by, or a combination of this glycoside with the tissue of the liver. An extraction of the rat liver slices gave a solution of marked cardiac activity. Actually the 15 cc. of supernatant fluid originally containing 1.5 mg. of g-strophanthin, showed an activity about equal to a 1:25,000 solution of g-strophanthin. The volume of the liver extract was 43 cc. and had an activity equal to a 1:50,000 solution of g-strophanthin. Thus about 1.46 mg. of g-strophanthin could be



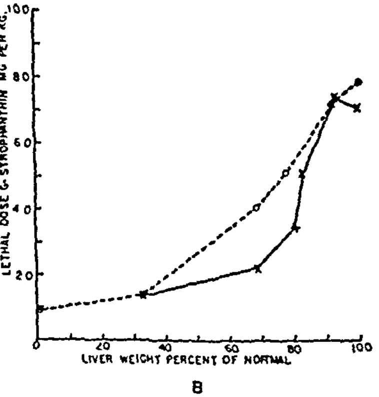


FIG. 2a. A comparison of liver hypertrophy and increase in the lethal dose of g-strophanthin following partial hepatectomy.

0—0—0 Liver weight per cent of normal.
X—X—X Lethal dose of g-strophanthin per cent of normal.

FIG. 2b. The relationship of percentage liver weight and lethal dose of g-strophanthin in the rat.

0—0—0 Acute partial hepatectomy.
X—X—X Liver hypertrophy.

TABLE 3

The elimination of g-strophanthin in the intact anaesthetised rat

NUMBER OF RATS	WEIGHT IN GRAMS			RATE OF ADMINISTRATION OF G-STROPHANTHIN				LETHAL DOSE OF G-STROPHANTHIN				EXPERIMENTAL TIME IN MINUTES				RATE OF ELIMINATION
	Average	Maximum	Minimum	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	
				mg.* per kg. per hour				mg. per kg.								mg. per kg. per hour
4	151	157	146	169.25	175.12	161.9	3.62	32.69	57.32	20.21	8.38	11.5	20	7	8.92	
5	221	244	219	114.24	121 15	108.16	2.333	31.76	66.74	18.55	8.84	16.7	35	9.5	9.85	
4	190	193	185	71.44	74.70	70.55	0.67	52.54	77.67	34.86	6.49	44	66	28	6.88	45.67
10	209	256	154	54.43	57.70	51.63	0.65	77.17	107.90	37.50	7.939	85.4	121	39	9.16	35.69
4	186	190	185	45.15	47.63	44.01	0.31	148.12	218.94	82.43	12.35	196	276	111	20.23	38.49

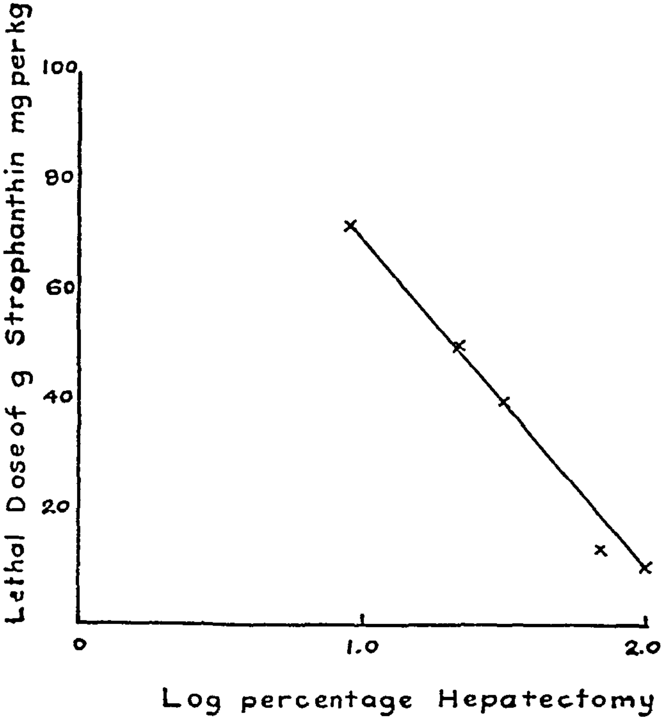


FIG. 1. The relationship of log percentage hepatectomy to the lethal dose of g-strophanthin in the rat.

TABLE 2

The effect of liver regeneration on the lethal dose of g-strophanthin in the rat

NUMBER OF RATS	WEIGHT IN GRAMS			RATE OF ADMINISTRATION OF G-STROPHANTHIN				LETHAL DOSE OF G-STROPHANTHIN				EXPERIMENTAL TIME IN MINUTES				LIVER WEIGHT PER CENT OF NORMAL				DAYS AFTER PARTIAL HEPATECTOMY
	Average	Maximum	Minimum	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	Average	Maximum	Minimum	Standard error	
				mg. per kg. per hour				*mg. per kg.*												
8	217	260	148	53.93	56.22	51.72	0.521	13.65	19.48	4.43	1.537	15.1	21	5.5	1.566	32.8	37.0	29.1	0.572	0
8	178	230	140	53.99	57.38	52.62	0.562	22.09	27.19	12.19	1.795	24.5	31	14	2 04	68.6	80.3	51.2	3.313	2
5	157	185	113	53.86	56.01	52.15	0.674	34.58	48.44	26.62	3.823	36.4	53	30	4.178	80.1	91.3	70.4	4.049	4
8	202	244	153	54.58	56.65	52.12	0.493	50.64	80.86	27.2	6.731	55.9	90	30	7 289	82.6	89.4	67.8	3.542	7
7	174	203	147	54.43	56.74	52.51	0.633	76.76	117.19	40.12	9.064	85.0	132	45	10.162	92.8	105.8	85.5	3.213	14
6	175	200	146	54.79	58.09	52.45	0.707	75.10	104.91	38.07	7.792	82.8	120	40	9.468	99.5	106.5	90.0	4.412	21

Since the liver plays such an important rôle in the fate of g-strophanthin, we investigated whether liver slices can destroy this glycoside. From table 6 it is clear that within 3 hours a reduction of the concentration of g-strophanthin in

In a number of experiments we tried to study some factors influencing this combination. The method used was the same as that described above, only HCN was added to make a M/200 solution. In another set of experiments nitrogen instead of oxygen was passed through the solution, otherwise the conditions were the same as those of the previous experiments. In both these instances combination to rat liver slices was markedly inhibited. It must be concluded that the process of combination between rat liver and g-strophanthin is dependent on an adequate oxygen supply.

In three experiments it was possible to demonstrate large quantities of a cardioactive substance in the bile following the infusion of g-strophanthin into the rat. From figure 3 it can be seen that whereas a 1:125 dilution of normal rat bile had no appreciable action on the contractions of the frog heart (figure 3a) the same dilution of bile collected during the infusion of g-strophanthin into the rat had a very marked cardiac activity (figure 3b). It has been possible to show that within 2–4 hours nearly 80–85 per cent of the infused g-strophanthin can be accounted for in the bile (table 7). Whether a greater percentage of the glycoside can be recovered by prolonging the period of bile collection, could not be decided by the present method since the concentration of g-strophanthin in the bile becomes so low that it cannot be determined quantitatively on the frog heart.

TABLE 6

The ability of rat liver slices to combine with g-strophanthin

3.55 grams of liver slices added to 15 cc. of 1:10,000 g-strophanthin in Krebs solution and placed in water bath at 38° C O_2 bubbled through solution. Extracts prepared as described under methods.

	NUMBER OF HEARTS TESTED	NUMBER OF HEARTS SHOWING EFFECT*					TIME FOR MAXIMAL EFFECT IN MINUTES	NUMBER OF HEARTS SHOWING RECOVERY
		+++	++	+	±	–		
-Strophanthin 1:150,000 in Locke solution	8			1	7		38	None
0 Time	7			1	6		33	None
3 Hours	6	1	5				39	3
6 Hours	6	2	1	3			37	5
9 Hours	6	1	2	3			33	3
Extract of liver slices	6					6	14	None
Extract of the supernatant fluid	5		3	2			40	3

* See footnote in table 5.

DISCUSSION. It has been shown that g-strophanthin elimination can be markedly influenced by partial and complete hepatectomy. Furthermore, the data presented show that a cardioactive substance appears in the bile of the rat following the injection of g-strophanthin into this animal. From the data of Hatcher (3), Hatcher and Eggleston (4) and our own, the process of elimination of g-strophanthin in the rat is one of fixation to the liver tissue followed by an excretion via the bile duct into the intestine. This is followed by a certain amount of inactivation and an excretion of this glycoside in the feces of the rat.

accounted for in the two solutions. Four other experiments conducted in a similar manner gave essentially the same results. It must be concluded that within the accuracy of our method no destruction of g-strophanthin by rat liver slices could be demonstrated. However, rat liver seems to show a marked ability

TABLE 4

The rate of elimination of g-strophanthin in the eviscerate rat

NUMBER OF RATS	WEIGHT IN GRAMS			RATE OF ADMINISTRATION OF G STROPHANTHIN				LETHAL DOSE OF G STROPHANTHIN				EXPERIMENTAL TIME IN MINUTES				RATE OF ELIMINATION
	Average	Maximum	Minimum	Average	Maximum	Minimum	Standard Error	Average	Maximum	Minimum	Standard Error	Average	Maximum	Minimum	Standard Error	
				mg per kg per hour				*mg per kg.*								*mg per kg per hour*
7	221	257	166	54.10	57.08	51.84	0.66	9.44	11.83	7.61	0.46	10.6	13	8	0.77	
3	206	215	200	44.14	46.14	43.73	0.58	8.73	9.62	8.07	0.39	11.9	13.2	10.5	0.72	
3	205	217	193	31.13	33.06	29.01	0.41	8.19	9.09	6.73	0.56	15.7	17.25	13.5	0.80	
3	192	193	191	22.68	23.21	22.32	0.52	9.14	9.31	9.01	0.42	24.2	25	23.5	0.71	6.70?
3	246	254	236	12.62	13.08	11.84	0.39	10.07	11.34	7.85	0.71	48	56	36	1.14	2.32
4	204	220	185	8.83	9.31	8.54	0.61	11.37	15.21	8.51	0.83	77.2	105	58	2.30	2.67

TABLE 5

The effect of rat blood on g-strophanthin

g-Strophanthin 1:10,000 in defibrinated rat blood diluted 1:1 with Krebs solution. Temperature of water bath 38.2° C. Oxygen bubbled through solution. At given time intervals 2 cc. of the solution was diluted to 30 cc. with cold blooded Krebs solution and tested on isolated frog hearts.

SOLUTION	NUMBER OF HEARTS TESTED	NUMBER OF HEARTS SHOWING EFFECT*					MAXIMUM EFFECT IN MINUTES	NUMBER OF HEARTS SHOWING RECOVERY
		+++	++	+	±	−		
0 Time . . .	8			2	6		32.5	2
5 Hours. .	8			1	7		31	1
9 Hours .	8			1	7		34	1

* +++ No effect on frog heart.
++ Reduction of amplitude from 1–30 per cent.
+ Reduction of amplitude from 31–60 per cent.
± Reduction of amplitude from 61–90 per cent or the appearance of irregularities.
− Complete systolic standstill.

to combine with this glycoside. From the above mentioned data one gram of rat liver must have combined with approximately 0.24 mg. of g-strophanthin. The treatment of the supernatant fluid in a manner similar to that of the liver slices did not increase the activity of this solution (table 6). The supernatant fluid, therefore, did not contain the material which can combine with g-strophanthin.

Elimination of g-strophanthin through the kidneys has been demonstrated (4); however, this process is much less important than hepatic elimination. Hatcher and Eggleston (4) are of the opinion that in the rabbit the elimination of cardiac glycosides is similar to that described for the rat. The experimental evidence for the rabbit is rather meager and further experiments will be necessary before any definite conclusions can be drawn.

Very little is known regarding the mechanism of elimination of g-strophanthin in the dog and cat. The possibility of an hepatic elimination in our opinion has not been excluded.

SUMMARY

In rats under anaesthesia, the lethal dose of g-strophanthin is reduced by partial or total (functional) hepatectomy (evisceration). This reduction is proportional to the log of percentage hepatectomy.

Liver hypertrophy following removal of the median and left lateral lobes of the liver is accompanied by a gradual increase in the lethal dose of g-strophanthin. This increase is not directly proportional to the increase in liver weight.

In *in vitro* experiments with rat blood and rat liver slices no appreciable destruction of g-strophanthin can be demonstrated.

Rat liver slices combine with g-strophanthin. This combination is inhibited by HCN and does not occur in the absence of oxygen.

The elimination of g-strophanthin is markedly reduced by evisceration.

Following the infusion of g-strophanthin appreciable quantities of cardioactive material can be detected in the bile of the rat.

In the light of previous experimental findings and our own data, the mechanism of g-strophanthin elimination has been discussed, and it is concluded that in the rat the hepatic route of excretion is the main route of elimination of g-strophanthin.

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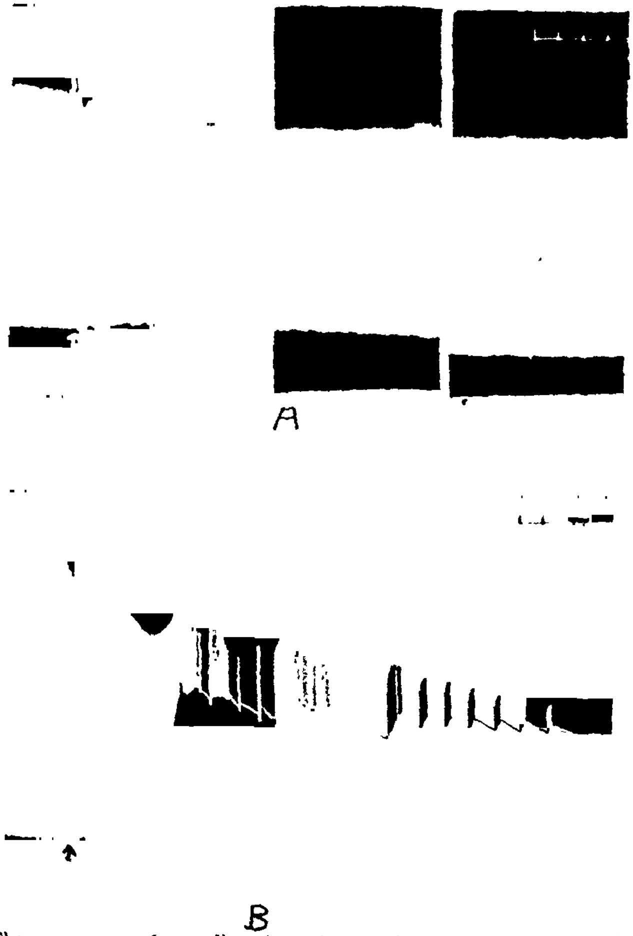

FIG. 3. The appearance of a cardioactive substance in the bile of the rat following the intravenous infusion of g-strophanthin.

A. The effect of normal rat bile diluted 1:125 with Locke solution on the isolated frog heart. *Each interval equals 15 minutes.*

B. The effect on the frog heart of rat bile diluted 1:125 with Locke solution and collected during the infusion of g-strophanthin into the rat.

Time in minutes. At arrow, introduction of diluted bile into the frog heart cannula.

TABLE 7

The recovery of g-strophanthin from the bile of the rat after its intravenous administration

EXPERIMENTAL NUMBER	RATE OF ADMINISTRATION OF G-STROPHANTHIN	MG G-STROPHANTHIN INFUSED	PERIOD OF BILE COLLECTION IN *MINUTES*	MG. OF G-STROPHANTHIN RECOVERED *IN THE BILE*	PERCENTAGE RECOVERY
	mg. per kg. per hour				
1	24.85	4	120	3.5	87
2	28.96	6	240	5.1	85
3	30.12	4	180	3.2	80

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Following the infusion of g-strophanthin appreciable quantities of cardioactive material can be detected in the bile of the rat.

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THE EFFECT OF SOME ACRIDINE COMPOUNDS ON THE GROWTH AND RESPIRATION OF E. COLI

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Albert and his co-workers (1) have made an extensive study of the antiseptic activity of a number of amino acridines. They have shown that there is a good correlation between the dissociation constant of a compound in this series and its bacteriostatic activity and postulate that they act as cationic antiseptics, that is, they combine with carboxyl groups on essential enzyme systems in the bacterial cell. There have been a few other studies (2-4) on the bacteriostatic action of various acridines but the effect of these drugs on the metabolic activity of bacteria has not been investigated. Through the courtesy of Dr. Albert we have obtained six amino acridines and have studied their effect on growth and on the oxidation of certain substances in order to determine whether there is a correlation between the two effects.

Experimental. E. coli, National Type Culture No 6522, was used for all the experiments since it grows readily in a synthetic medium of the following composition: 2.0 gm glucose, 4.0 gm NaCl, 2.0 gm KH_2PO_4, 2.0 gm $(NH_4)_2HPO_4$, 0.2 gm $MgSO_4$ per liter. The pH was adjusted to 7.2 and medium was autoclaved 10 minutes. The growth was measured in this medium by following the increase in density by means of the Evelyn photo colorimeter. This method has been shown (5) to give an accurate measure of growth and the effect of the drugs on the light transmission can easily be compensated for. The metabolic experiments were carried out in the usual Warburg apparatus. The bacteria were grown for 16 hours in the synthetic medium and then centrifuged and washed once with water. They were then suspended in M/20 phosphate buffer either pH 7.8 or 6.7 and adjusted to a standard density. This suspension with various concentrations of the drugs was put in the Warburg vessels and after temperature equilibrium had been obtained 2.0 mg. of substrate were added from the side arm. Five substrates were used, glucose, pyruvic acid, lactic acid, asparagin and oleic acid.

Fig. 1 shows the effect of the drugs on the growth. The values were taken when the growth of the control was half way through the logarithmic phase. The order of activity is the same as that found by previous workers. 3-amino- and 5 amino-1,2,3,4,tetrahydro-acridine have very little effect even when the inoculum is very small. 2,8 diamino- and 2,7 diamino-acridine are the most effective drugs at low concentrations, followed by 5 amino and 2 dimethyl-7-amino acridine. Fig. 2 shows the effect of the size of the inoculum on the subsequent action of the same concentration of the drugs. The two drugs that are least effective do not show any significant change, when the inoculum is halved and are still without effect when 0.1 cc. of inoculum is used. The inhibition by 2 dimethyl-7-amino acridine when the control is half way through the logarithmic growth phase is 52% with 0.3 cc. inoculum and 43% with 0.6 cc. inoculum. Of the three drugs that completely inhibit growth for 8 hours with 0.3 cc. inoculum all allow some growth beginning at about 3 hours, when the larger inoculum is used, but in the case of 2,8 diamino acridine the growth is negligible. The action of the drugs is very little affected by presence of peptone in the medium and comparatively large quantities of riboflavin fail to antagonize their bacteriostatic action.

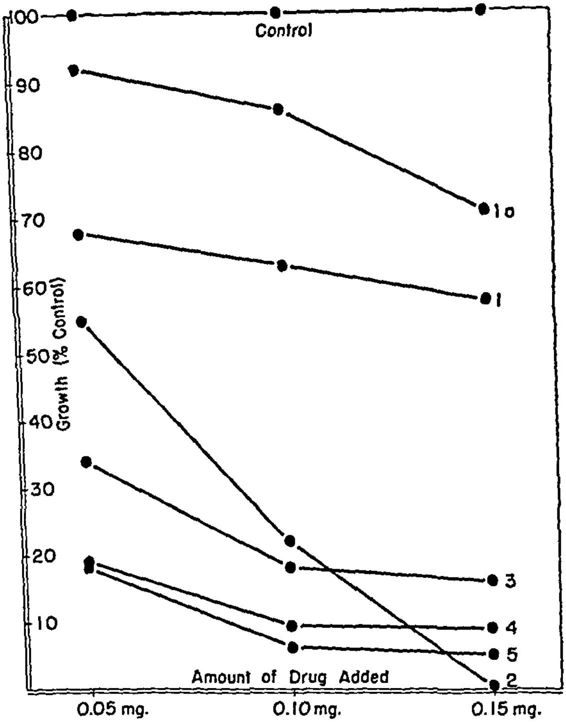

FIG. 1. The effect of 3 concentrations of several acridine compounds on growth. The figures represent the growth when the control was halfway in its logarithmic growth phase. The concentrations are given as amounts added to 7.0 cc. of media. 1 = 3-amino-; 1a = 5-amino-1, 2, 3, 4, tetrahydro-; 2 = 2-dimethyl-7-amino-; 3 = 5-amino-; 4 = 2,7-diamino-; 5 = 2, 8-diamino-acridine.

Once the order of activity on growth was established, the effect of the drugs on the oxidation of various substrates was tested to determine whether they acted in the same order and at comparable concentrations. The results are shown in fig 3. The drugs are numbered in the order of effectiveness on growth, i.e., No. 1 is least effective, No. 5 most. (No. 1a which was actually the least effective was not available in sufficient amounts to use in the metabolic experiments.) The following generalizations can be made. No. 1, 3-amino-acridine, which is least effective on growth is also the least effective inhibitor of the oxidation of the sub-

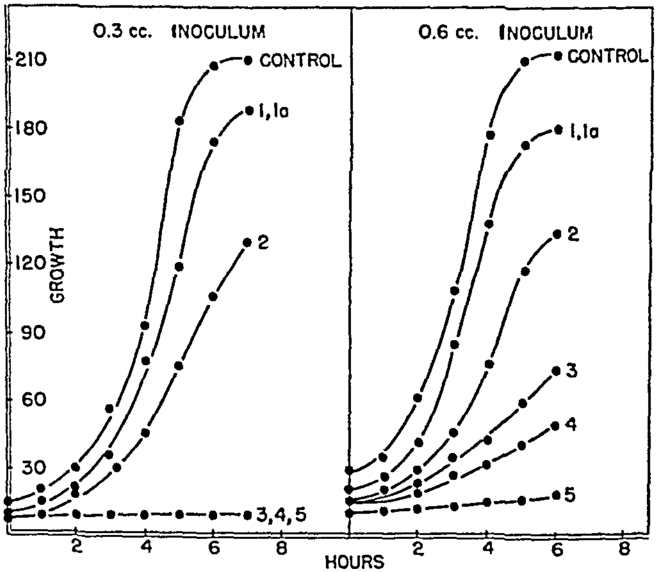

FIG. 2. The effect of the size of the inoculum on the inhibition of growth by various acridine compounds. 0.1 mg. of each drug was added to 7.0 cc. of media. (See fig. 1 for key.)

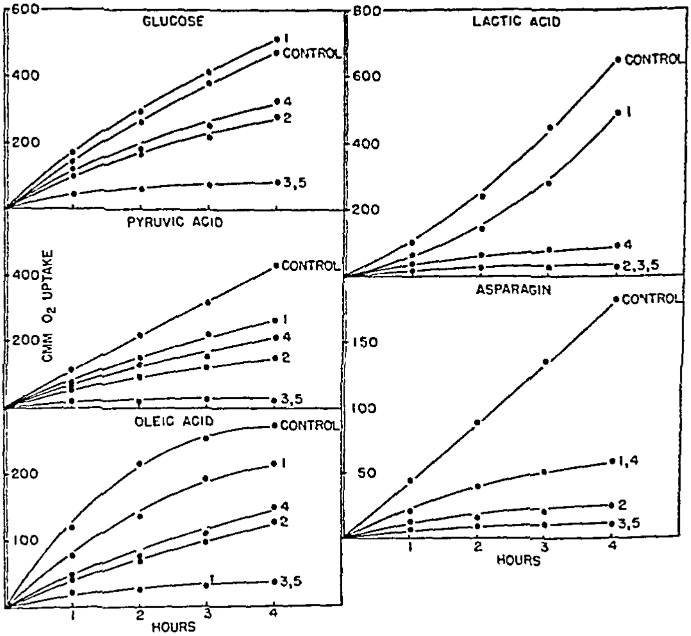

FIG. 3. The effect of 0.1 mg./cc. of various acridine compounds on the oxidation of 2.0 mg. of each of the substrates, pH 7.8, 37°. (See fig. 1 for key.)

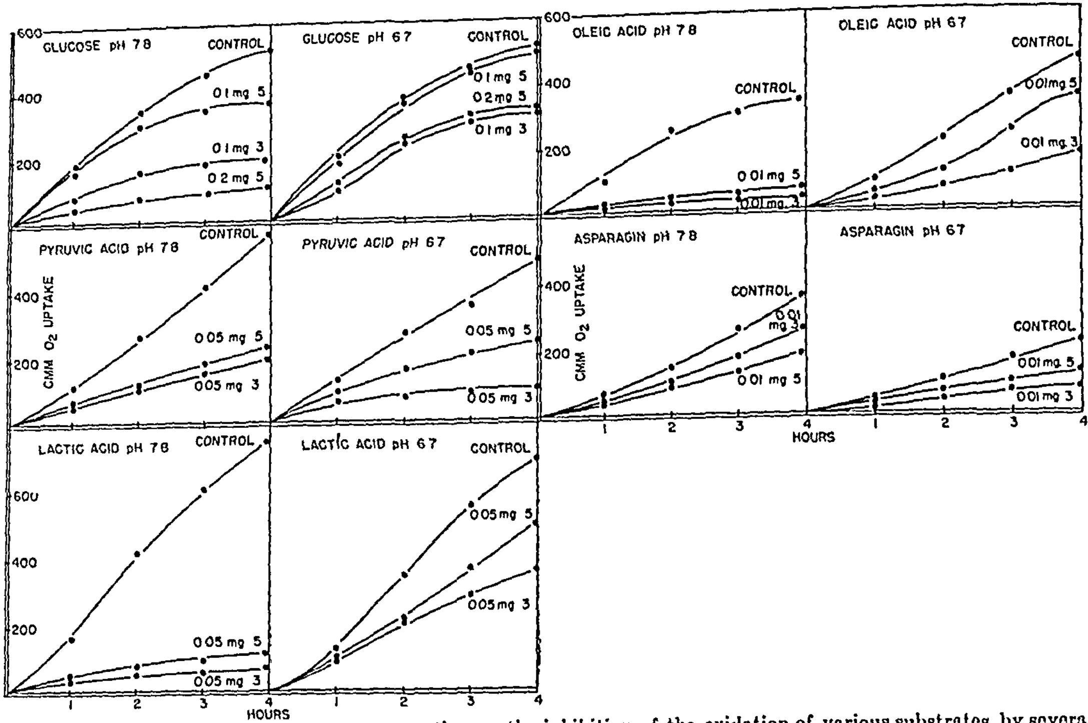

FIG. 1. The effect of hydrogen ion concentration on the inhibition of the oxidation of various substrates by several concentrations of 5-amino-acridine (3) and 2,8-diamino-acridine (5). The concentrations are given in mg./2.0 cc.

strates. Nos. 3 and 5 are consistently the best metabolic inhibitors, but as shown in fig. 4, which gives the results with lower concentrations of the two drugs, No. 3 is more effective than No. 5. For all substrates No. 4 inhibits less than No. 2. There is thus no correlation between the order of effectiveness on growth of these drugs, which increases from No. 1–5 and the order of effectiveness on oxidative metabolism of five typical substrates which is as follows: Nos. 1, 4, 2, 5, 3.

Fig. 4 also shows that the percentage inhibition in all cases decreases in acid. This is to be expected for cationic antiseptics and confirms Albert's hypothesis concerning the mode of action of acridine compounds. It is also evident that the oxidations of the various substrates differ in their sensitivity to the drugs. This is illustrated in fig. 4 from which it is possible to make the following calculations for No. 3, 5-amino-acridine. It inhibits growth 66% when present in a concentration of 0.007 mg./cc. At pH 7.8 the oxidation of glucose at 3 hours in inhibited 60% by 0.05 mg./cc.; that of pyruvic acid, 55% by 0.025 mg./cc.; that of lactic acid, 88% by 0.024 mg./cc.; that of oleic acid, 94% by 0.005 mg./cc.; and that of asparagin, 35% by 0.005 mg./cc. With the exception of glucose, the oxidation of the other substrates is effectively inhibited by concentrations of the drug which inhibit growth despite the fact that more bacteria are present in the Warburg vessels than are initially present in the growth tubes.

DISCUSSION. A drug may inhibit bacterial growth by interfering with the anabolic or catabolic metabolism of the cell. In the former cases growth would be inhibited directly, in the latter, indirectly, by inhibiting oxidation which supplies the energy for the synthesis of protoplasmic constituents. In the case of the sulfa drugs and possibly also penicillin oxidations are not inhibited by concentrations of the drug which are bacteriostatic. Presumably, therefore, they are interfering directly with some anabolic reactions. A number of other antiseptics are known to inhibit oxidations in concentrations comparable to those used to inhibit growth. The question arises whether their bacteriostatic action can be explained on this basis. It was for the purpose of answering this that the present study of the acridine compounds was undertaken. These compounds inhibit the oxidation of typical substrates in low concentrations which is presumptive evidence that this is the mechanism by which they inhibit growth. Since, however, there is no correlation between their order of activity on growth and on oxidation, it is necessary to conclude that this mechanism is not the principal one by which bacteriostasis is achieved, although it may be an important subsidiary one. It is also probable that these drugs are not interfering with the utilization or formation of riboflavin for its addition to the medium has no effcte on the growth inhibition.

SUMMARY

1) The order of activity of several acridine compounds on the growth of E. coli on a synthetic medium has been determined.

2) Certain of the compounds in concentrations comparable to those that produce bacteriostasis inhibit the oxidation of glucose, pyruvic acid, lactic acid, asparagin and oleic acid.

3) The order of activity on the oxidative reactions is, however, different from that on growth.

4) Riboflavin does not antagonize the bacteriostatic action of these compounds.

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THE ABSORPTION OF SULPHONAMIDES IN MICE

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Several authors have recently studied the absorption and distribution in experimental animals of groups of sulphonamides, chosen rather for the purpose of relating some chemical or physical property to pharmacological behaviour than for therapeutic interest (1, 2). Similar work has been carried out in these laboratories over a number of years, and it is hoped to present some of the results in a series of papers, of which this, dealing with a number of drugs in common use, is the first.

Initially, in an attempt to economise in drug usage, especially where the compounds were not available in quantity, we obtained our preliminary data from absorption experiments in the mouse. It soon became apparent that the blood concentrations attained by the same sulphonamide in different groups of mice varied considerably, and that large numbers of animals must be used if valid comparisons were to be made between drugs of closely related structure. Further, it was thought desirable that the technique adopted should permit statistical analysis of the results. Previous workers (2, 3, 4, 5, 6, 7, 8, 9, 10, 11) have neglected these aspects of the problem. Results have frequently been based on the use of 6 or fewer animals, and a sacrificial technique has often been employed; that is, a group of animals was killed and heart blood pooled for each point on the absorption curve (3, 5, 11). Such a technique does not give a smooth curve, and is unnecessary now that sulphonamides can readily be determined in 0.02 ml. of blood. This paper presents smooth mean absorption curves of sulphanilamide, sulphapyridine, sulphathiazole, sulphaguanidine, sulphadiazine, sulphamezathine and sulphamerizine, which are based on at least 30 animals in each case. In addition the three main features of an absorption curve, the maximum blood level, the time at which this is attained, and the rate of disappearance from the blood, have been calculated and significant differences between drugs as regards each of these determined by statistical analysis. The results serve as standards for the preliminary examination of novel drugs.

EXPERIMENTAL. The techniques described are those which have been employed throughout our work on sulphonamides, and they are given here in sufficient detail to avoid repetition in later publications.

Three albino mice (20–25 gm.) received 5 mgm. of drug 20 gm., administered by stomach tube as a 1% solution of the sodium salt. When the sodium salt could not be made, or was insufficiently soluble, a 1% solution of the hydrochloride (e.g., of sulphaguanidine and its derivatives), or a 1 per cent dispersion, was used. Pooled tail blood (0.02–0.04 ml.) was withdrawn at intervals of 0.20, 0.40, 1.00, 1.30, 2.30, 3.30, 5.00, 7.00 and 24.00 hours, and free drug determined therein by the micro method of Rose and Bevan (12). Animals were allowed to eat and drink freely before and throughout the experiment. The particular dose was employed because the blood concentrations attained approximated to those encountered clinically. In all experiments the assumption has been made that the substance assayed was identical with the administered drug. The validity of this assumption has not been checked by unequivocal analysis, but previous experience in this field has been that most sulphonamides are metabolised to a slight extent only, except by acetylation. In the present series of drugs the individual experiment was repeated 10 times (30 mice) with sulphanilamide, sulphapyridine, sulphathiazole and sulphaguanidine and 15 times (45 mice) with each of the three sulphapyrimidines.

Results. The mean blood concentration-time curves of the seven drugs, given in diagrams 1, 2 and 3, show obvious differences as regards blood concentration, rate of absorption, and rate of disappearance from the blood. The significance of these differences has been assessed by statistical analysis.

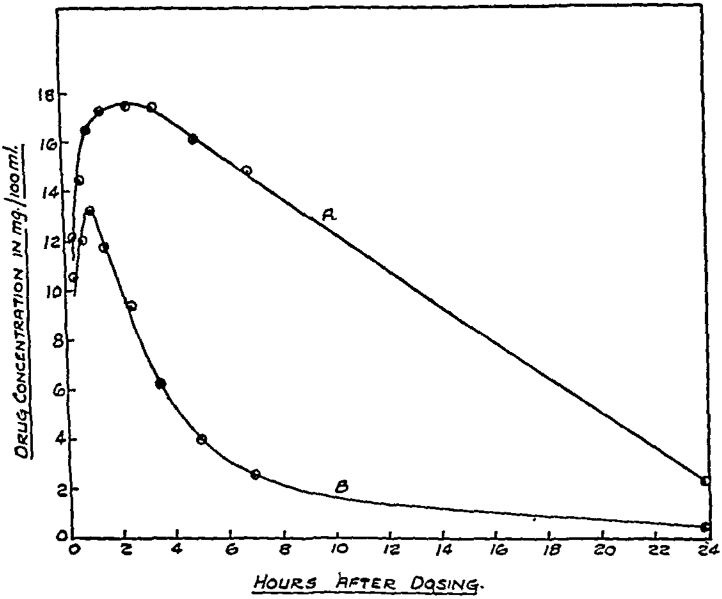

Diagram 1. Mean Blood Concentration/Time Curves of Sulphonamides in Mice Following Single Oral Doses of 5 mgm./20 grams
A. Sulphadiazine. B. Sulphanilamide

Smooth individual curves were drawn freehand, and the maximum blood concentration and the time taken to attain this measured. Defining the rate of disappearance of the drug from the blood was more difficult. An arbitrary expression, the time (C.7) required for the blood concentration to fall from that attained 7 hours after dosing to two-thirds of this figure, has been adopted. In the case of drugs which are rapidly eliminated we select for similar treatment the levels attained after a shorter period than 7 hours, for example 3½ or 5 hours, giving values signified by C.3½ or C.5, respectively. Comparison of drugs which

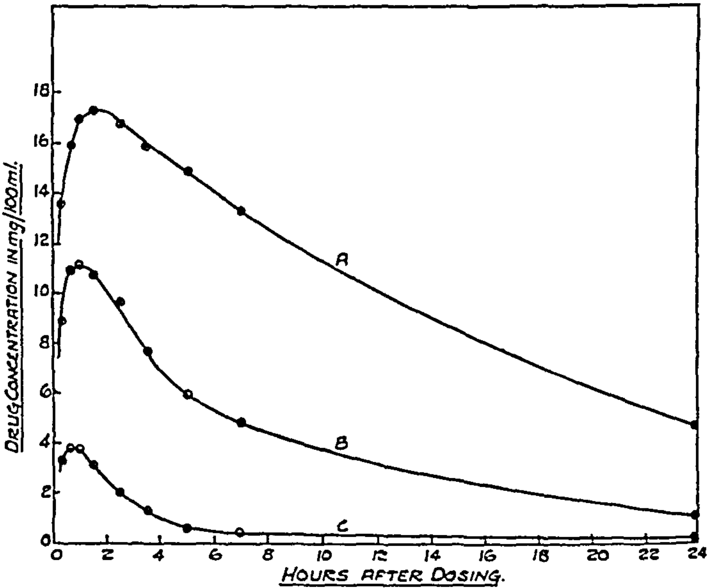

DIAGRAM 2. MEAN BLOOD CONCENTRATION/TIME CURVES OF SULPHONAMIDES IN MICE FOLLOWING SINGLE ORAL DOSES OF 5 MGM./20 GRAMS

A. Sulphamerizine. B. Sulphapyridine. C. Sulphaguanidine

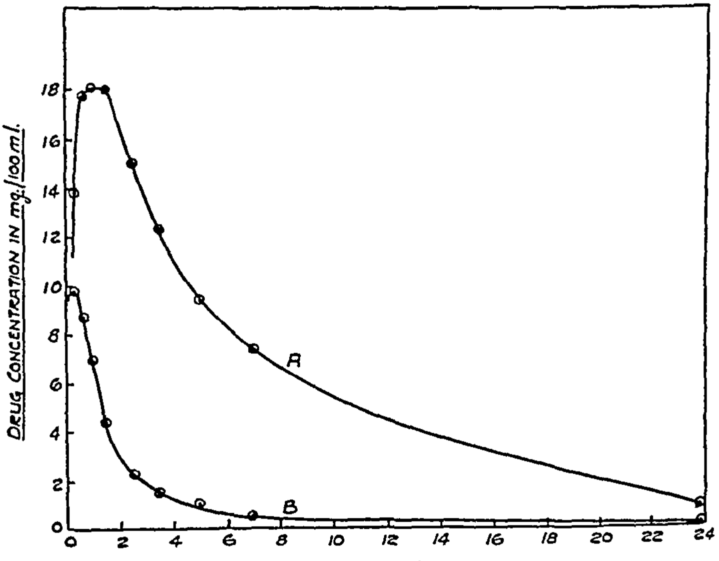

DIAGRAM 3. MEAN BLOOD CONCENTRATION/TIME CURVES OF SULPHONAMIDES IN MICE FOLLOWING SINGLE ORAL DOSES OF 5 MGM./20 GRAMS

A. Sulphamezathine. B. Sulphathiazole

are removed from the blood stream at widely different rates is not entirely satisfactory but has been carried out here in order to show the technique used. A value thus obtained is of course a function of several variables, for example, clearance by the kidney, excretion into the intestine, and metabolism. It may also be affected by the continuation of absorption during part of the time of its measurement. However, it provides a valuable index of the retention of free drug in the blood following oral administration in a species that, like man, possesses effective acetylation mechanisms; as such it has proved useful in the comparison of drugs of high persistence.

TABLE 1

Blood levels of sulphanilamide in the mouse

EXPT. NO.	BLOOD LEVELS IN MGM./100ML. AFTER									MAX.	T MAX.	C7
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hr.*
1	9.0	12.5	11.0	9.0	5.8	3.8	2.4	1.5	0.3	11.1	50	2.1
2	7.2	9.9	10.8	7.9	8.7	5.7	3.2	4.3	1.4	10.9	70	5.0
3	10.3	12.3	12.9	12.5	10.0	7.0	3.4	1.7	0	13.0	70	1.2
4	11.6	12.4	12.8	12.7	10.2	6.5	3.1	0.5	0	13.0	70	0.7
5	11.6	14.0	16.0	13.5	9.8	4.6	4.8	2.9	0.3	16.0	60	2.0
6	11.7	12.0	12.8	12.1	6 9	3.7	2.6	1.7	0.5	12.8	60	1.7
7	12.0	13.8	16.3	13.8	9.7	7.0	3.7	3.0	0.2	16.3	60	1.4
8	16.4	14.6	15.0	15.1	12.7	8.9	7.7	4.1	0.5	15.1	75	1.5
9	8.0	10.0	15.5	10.2		6.7	2.4	2.1	0.4	10.3	75	1.8
10	7.5	8.6	8.8	10.7	9.9	8.0	6.7	3.7	0.3	10.2	100	2.0
Means	10.5	12.0	13.2	11.75	9.3	6 2	4.0	2.55	0.4	12 9	70	1.95
Standard deviation .										2.3	13.5	1.2
Standard error of mean ..										0.8	4.2	0.35

The set of values from repeated experiments with a single drug gave a mean value whose accuracy could be estimated by statistical analysis, and which could be compared with the corresponding mean of another drug. Tables 1 to 7 give the blood concentrations recorded in individual experiments, the characteristic values measured from individual curves, and the means of these, together with standard deviations from and standard errors of the means.

Comparison of drugs. The significance of a difference between the mean values for a pair of compounds was determined by the usual method of the "t" test (13), based on the variation in the given value between the animals of the same group. Probability levels of 0.05 and 0.01 are regarded as significant and decisive, respectively. In all cases the plus and minus limits of error for a mean value correspond to the 0.05 level of probability, i.e., there is only one chance in twenty of the error exceeding the given limit.

A. *Speed of absorption.* The time of attaining the maximum blood concen-

TABLE 2

Blood levels of sulphapyridine in the mouse

EXPT. NO.	LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C7
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hr.*
1	5.2	7.8	7.9	8.2	9.3	5.7	4.7	5.8		8.3	90	2.5
2	14.6	15.0	11.0	12.9	13.0	8.1	7.5	7.2	1.3	14.0	80	5.4
3	12.6		12.3	17.0	14.0	10.9	9.4	8.1	2.2	16.6	65	5.7
4	12.4	13.6	18.0	16.2	14.1	11.0	7.7	6.5	1.9	16.5	65	6.1
5	6.8	6.6	7.4	7.2	7.6	6.9	4.8	2.0	1.0	7.7	60	3.0
6	5.6	6.3	7.1	5.2	6.4	6.1	4.3		1.5	7.1	80	6.6
7	11.4	15.9	13.4	11.1	8.6	5.4	3.6	3.4	0.4	15.8	35	2.6
8	5.1	6.7	7.7	10.0	6.0	5.8	4.2	1.8	0.2	7.8	75	2.3
9	6.0	13.2	11.8	8.4	7.4	6.9	6.1	4.3	0.7	13.3	30	4.2
10	9.6	12.7	14.3		10.1	9.4	6.4	4.0	0.5	13.2	40	3.0
Means...	8.9	10.9	11.1	10.7	9.65	7.6	5.9	4.8	1.1	12.0	63	4.15
Standard deviation..										3.9	20.7	1.7
Standard error of mean..										1.3	6.9	0.55

TABLE 3

Blood levels of sulphathiazole in the mouse

EXPT. NO.	LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C5
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hr.*
1	4.4	5.8	7.0	2.9	5.5	4.7	2.8	2.1	0	7.2	70	2.0
2	10.8	6.6	6.0	2.9	1.5	1.4	0.2		0	10.8	20	0.7
3	3.3	4.0	4.7	2.9	1.4	0.6	0.7	0.5	0	4.1	30	0.7
4	13.8	11.1	8.7	5.3	2.1	1.7	0.9	0.8	0.2	14.0	10	1.7
5	20.9	17.5	10.7	5.3	2.3	0.8	0.5	0.5	0.1	21.2	10	0.8
6	10.8	10.3	7.6	6.1	2.0	2.0	1.3	0.1	0.2	10.9	15	0.6
7	7.4	7.7	6.8	4.9	1.8	0.5	0.6	0.2	0	7.8	30	0.6
8	10.8	9.5	8.4	5.7	2.7	1.4	2.0	1.0	0.4	10.9	10	1.5
9	8.3	7.2	4.2	3.8	1.1	0.7	0.5	0	0	8.5	10	1.1
10	7.0	6.8	4.9	4.3	1.6	0.8	0.6	0	0	7.0	25	0.5
Means...	9.75	8.65	6.9	4.4	2.2	1.5	1.0	0.5	0.1	10.2	23	1.0
Standard deviation..										4.7	10.8	0.5
Standard error of mean..										1.6	3.6	0.2

tration is regarded as the criterion of speed of absorption. In the following series drugs are arranged in order of decreasing speed of absorption, the times in minutes at which maximum concentrations were attained and limits of error

TABLE 4

Blood levels of sulphaguanidine in the mouse

EXPT. NO.	BLOOD LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C5
	20 min.	40 min.	1 hr.	2½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hrs.* *
1	3.4	3.9	2.8	2.6	1.5	1.6	0.6	0.2	0	3.6	40	0.8
2	2.9	5.0	4.6	4.9	2.7	1.6	0.6	0.3	0	5.2	70	1.8
3	3.7	4.2	4.7	3.1	2.0	1.4	0.7	0.7	0.7	4.4	50	3.0
4	4.0	5.8	5.6	3.7	2.5	2.5	0	0	0	5.9	50	
5	4.8	4.3	4.8	2.8	2.1	0.9	0.3	0	0	4.7	40	0.5
6	5.7	2.9	1.7	1.1	0.5	0.5	0.3	0.2	0	5.7	15	2.0
7	1.5	2.6	2.9	2.7	2.5	1.0	0.6	0.5	0.1	3.0	70	2.0
8	2.8	2.6	2.9	2.6	1.4	1.2	0.9	0.8	0.2	2.9	60	2.8
9	1.7	3.8	4.2	3.8	3.1	1.5	1.5	0.8	0.5	4.2	60	2.1
10	2.9	3.2	3.1	3.4	1.8	1.3		1.7	0.5	3.2	40	4.5
Means...	3.3	3.8	3.7	3.1	2.0	1.35	0.6	0.5	0.2	4.3	50	2.2
Standard deviation......										1.1	16.8	1.2
Standard error of mean......										0.4	5.6	0.4

TABLE 5

Blood levels of sulphamezathine in the mouse

EXPT. NO.	BLOOD LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C7
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hr.*
1	7.8	12.6	14.0	11.5	12.2	9.7	6.1	4.0	0.4	14.2	40	2.5
2	12.4	18.2	21.0	23.0	21.0	15.2	13.1	7.3	1.4	23.1	100	3.0
3	9.4		13.6	13.4	12.7	12.2	8.5	7.7	2.2	13.3	100	6.5
4	10.3	13.8	11.1	12.9		10.0	7.8	7.0	0.9	14.2	30	5.0
5	20.5	17.9	19.9	18.4	10.4	7.4	6.5	5.9	0	19.9	60	4.0
6	15.8	27.2	24.0	21.2	19.2	14.6	15.2	10.3	2.6	21.9	40	5.3
7	12.6	16.6	20.2	27.5	18.2	15.2	10.6	8.3	0.4	20.8	90	3.7
8	14.7	18.0	18.2	16.0	13.6	11.4	6.0	4.5	0	18.2	50	2.5
9	13.8	20.3	18.2	21.4	14.7	11.5	7.1	7.0	0.8	20.0	70	3.4
10	20.5	24.7	21.0	21.9	15.6	12.8	7.8	7.0	0.2	22.2	60	4.5
11	18.9	24.9	22.7	22.0	18.7	15.5	10.0	5.8	0	22.7	60	2.3
12	12.7	12.7	16.3	14.2	10.9	11.7	9.8	8.6	0.8	14.6	70	5.0
13	8.9	8.2	13.9	12.4	12.0	9.7	9.4	7.0	1.0	12.5	90	5.0
14	13.3	16.2	18.0	17.0	15.4	14.0	12.5	11.4	0.2	17.0	50	4.8
15	13.6	16.7	17.6	17.5	16.0	13.3	10.8	8.7	0.7	17.6	80	5.2
Means...	13.7	17.7	18.0	18.0	15.0	12.3	9.4	7.4	0.8	18.1	65	4.2
Standard deviation......										3.7	22.3	1.25
Standard error of mean......										0.95	5.8	0.3

TABLE 2

Blood levels of sulphapyridine in the mouse

EXPT. NO.	LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C7
	20 min	40 min	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.	*mgm / 100 ml.*	*min.*	*hr.*
1	5.2	7.8	7.9	8.2	9.3	5.7	4.7	5.8		8.3	90	2.5
2	14.6	15.0	11.0	12.9	13.0	8.1	7.5	7.2	1.3	14.0	80	5.4
3	12.6		12.3	17.0	14.0	10.9	9.4	8.1	2.2	16.6	65	5.7
4	12.4	13.6	18.0	16.2	14.1	11.0	7.7	6.5	1.9	16.5	65	6.1
5	6.8	6.6	7.4	7.2	7.6	6.9	4.8	2.0	1.0	7.7	60	3.0
6	5.6	6.3	7.1	5.2	6.4	6.1	4.3		1.5	7.1	80	6.6
7	11.4	15.9	13.4	11.1	8.6	5.4	3.6	3.4	0.4	15.8	35	2.6
8	5.1	6.7	7.7	10.0	6.0	5.8	4.2	1.8	0.2	7.8	75	2.3
9	6.0	13.2	11.8	8.4	7.4	6.9	6.1	4.3	0.7	13.3	30	4.2
10	9.6	12.7	14.3		10.1	9.4	6.4	4.0	0.5	13.2	40	3.0
Means	8.9	10.9	11.1	10.7	9.65	7.6	5.9	4.8	1.1	12.0	63	4.15
Standard deviation										3.9	20.7	1.7
Standard error of mean										1.3	6.9	0.55

TABLE 3

Blood levels of sulphathiazole in the mouse

EXPT. NO.	LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C5
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.	*mgm / 100 ml.*	*min.*	*hr.*
1	4.4	5.8	7.0	2.9	5.5	4.7	2.8	2.1	0	7.2	70	2.0
2	10.8	6.6	6.0	2.9	1.5	1.4	0.2		0	10.8	20	0.7
3	3.3	4.0	4.7	2.9	1.4	0.6	0.7	0.5	0	4.1	30	0.7
4	13.8	11.1	8.7	5.3	2.1	1.7	0.9	0.8	0.2	14.0	10	1.7
5	20.9	17.5	10.7	5.3	2.3	0.8	0.5	0.5	0.1	21.2	10	0.8
6	10.8	10.3	7.6	6.1	2.0	2.0	1.3	0.1	0.2	10.9	15	0.6
7	7.4	7.7	6.8	4.9	1.8	0.5	0.6	0.2	0	7.8	30	0.6
8	10.8	9.5	8.4	5.7	2.7	1.4	2.0	1.0	0.4	10.9	10	1.5
9	8.3	7.2	4.2	3.8	1.1	0.7	0.5	0	0	8.5	10	1.1
10	7.0	6.8	4.9	4.3	1.6	0.8	0.6	0	0	7.0	25	0.5
Means	9.75	8.65	6.9	4.4	2.2	1.5	1.0	0.5	0.1	10.2	23	1.0
Standard deviation										4.7	10.8	0.5
Standard error of mean										1.6	3.6	0.2

tration is regarded as the criterion of speed of absorption. In the following series drugs are arranged in order of decreasing speed of absorption, the times in minutes at which maximum concentrations were attained and limits of error

TABLE 4

Blood levels of sulphaguanidine in the mouse

EXPT. NO.	BLOOD LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C5
	20 min.	40 min.	1 hr.	2½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hrs.* *
1	3.4	3.9	2.8	2.6	1.5	1.6	0.6	0.2	0	3.6	40	0.8
2	2.9	5.0	4.6	4.9	2.7	1.6	0.6	0.3	0	5.2	70	1.8
3	3.7	4.2	4.7	3.1	2.0	1.4	0.7	0.7	0.7	4.4	50	3.0
4	4.0	5.8	5.6	3.7	2.5	2.5	0	0	0	5.9	50	
5	4.8	4.3	4.8	2.8	2.1	0.9	0.3	0	0	4.7	40	0.5
6	5.7	2.9	1.7	1.1	0.5	0.5	0.3	0.2	0	5.7	15	2.0
7	1.5	2.6	2.9	2.7	2.5	1.0	0.6	0.5	0.1	3.0	70	2.0
8	2.8	2.6	2.9	2.6	1.4	1.2	0.9	0.8	0.2	2.9	60	2.8
9	1.7	3.8	4.2	3.8	3.1	1.5	1.5	0.8	0.5	4.2	60	2.1
10	2.9	3.2	3.1	3.4	1.8	1.3		1.7	0.5	3.2	40	4.5
Means...	3.3	3.8	3.7	3.1	2.0	1.35	0.6	0.5	0.2	4.3	50	2.2
Standard deviation										1.1	16.8	1.2
Standard error of mean										0.4	5.6	0.4

TABLE 5

Blood levels of sulphamezathine in the mouse

EXPT. NO.	BLOOD LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C7
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hr.*
1	7.8	12.6	14.0	11.5	12.2	9.7	6.1	4.0	0.4	14.2	40	2.5
2	12.4	18.2	21.0	23.0	21.0	15.2	13.1	7.3	1.4	23.1	100	3.0
3	9.4		13.6	13.4	12.7	12.2	8.5	7.7	2.2	13.3	100	6.5
4	10.3	13.8	11.1	12.9		10.0	7.8	7.0	0.9	14.2	30	5.0
5	20.5	17.9	19.9	18.4	10.4	7.4	6.5	5.9	0	19.9	60	4.0
6	15.8	27.2	24.0	21.2	19.2	14.6	15.2	10.3	2.6	21.9	40	5.3
7	12.6	16.6	20.2	27.5	18.2	15.2	10.6	8.3	0.4	20.8	90	3.7
8	14.7	18.0	18.2	16.0	13.6	11.4	6.0	4.5	0	18.2	50	2.5
9	13.8	20.3	18.2	21.4	14.7	11.5	7.1	7.0	0.8	20.0	70	3.4
10	20.5	24.7	21.0	21.9	15.6	12.8	7.8	7.0	0.2	22.2	60	4.5
11	18.9	24.9	22.7	22.0	18.7	15.5	10.0	5.8	0	22.7	60	2.3
12	12.7	12.7	16.3	14.2	10.9	11.7	9.8	8.6	0.8	14.6	70	5.0
13	8.9	8.2	13.9	12.4	12.0	9.7	9.4	7.0	1.0	12.5	90	5.0
14	13.3	16.2	18.0	17.0	15.4	14.0	12.5	11.4	0.2	17.0	50	4.8
15	13.6	16.7	17.6	17.5	16.0	13.3	10.8	8.7	0.7	17.6	80	5.2
Means...	13.7	17.7	18.0	18.0	15.0	12.3	9.4	7.4	0.8	18.1	65	4.2
Standard deviation										3.7	22.3	1.25
Standard error of mean										0.95	5.8	0.3

of these being given. Significant differences between adjacent compounds in the series are indicated by the interpolation between them of "s" (significant) or "d" (decisive).

Sulphathiazole 23 ± 8.0 (d); sulphaguanidine 50 ± 11.9; sulphapyridine 63 ± 14.7; sulphamezathine 65 ± 12.4; sulphanilamide 70 ± 9.2 (d); sulphamerizine 115 ± 17.3 (d); sulphadiazine 165 ± 30.5.

The difference between sulphaguanidine and sulphamezathine is not significant (p = 0.08), that between the former and sulphanilamide is decisive.

TABLE 6

Blood levels of sulphamerizine in the mouse

EXPT. NO.	BLOOD LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C7
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./ 100 ml.	*min.*	*hr.*
1	9.8	13.5	15.0	11.7	12.8	13.5	11.0	13.6	5.8	13.3	140	10.3
2	12.3	18.8	12.5	13.8	13.6	10.2	10.3	7.4	2.5	14.0	90	4.3
3	16.0		26.4	19.4	16.9	16.7	14.2	13.0	4.1	18.8	90	7.3
4	12.8	16.6	14.0	15.3	15.0	17.8	11.4	9.3	3.9	15.6	100	5.7
5	11.2	15.5	20.0	15.0	16.4	13.0	10.4	11.1	2.4	17.6	90	5.2
6	14.7	17.7	20.0	20.0	15.8	13.4	12.6	14.8	4.3	20.4	70	7.7
7	12.6	14.1	17.3	15.0	14.4	13.8	13.7	11.7	6.0	15.8	90	9.0
8	8.6	11.0	11.3	14.0	17.0	15.6	13.6	11.1	4.1	16.8	150	7.7
9	9.6	10.8	12.2	15.5	16.1	14.6	13.4	11.4	3.4	16.4	120	7.0
10	16.2	16.3	15.2	19.0	18.0	15.3	12.4	10.9	5.3	19.4	90	8.5
11	15.0	17.6	20.5	16.6	16.1	17.4	15.6	12.4	4.7	19.5	120	5.8
12	18.6	18.7	17.7	20 0		16.8	20.0	19.0	8.3	20.7	180	9.5
13	14.0	18.2	16.6	19.4	17.6	18.9	28.5	17.0	6.9	19.6	110	9.5
14	16.2	16.2	16.2	20.5	21.0	18.0	16.5	17.2	3.9	18.3	150	6.9
15	14.3	17.4	18.3	23.5	22.5	22.0	20.0	18.2	3.9	22.5	150	7.0
Means. .	13.5	15.9	16 9	17.2	16.7	15.8	14.9	13.2	4.6	17.9	115	7.4
Standard deviation										2.6	31.6	1.7
Standard error of mean . .										0.7	8.2	0.45

B. Maximum blood concentrations. The series of decreasing maximum blood concentration is given below. All concentrations are in mgm./100 ml.

Sulphamezathine 18.1 ± 2.0; sulphamerizine 17.9 ± 1.4; sulphadiazine 17.8 ± 1.2 (d); sulphanilamide 12.9 ± 1.7; sulphapyridine 12.0 ± 2.9; sulphathiazole 10.2 ± 3.6 (d); sulphaguanidine 4.3 ± 0.8.

The difference between sulphanilamide and sulphathiazole is not significant (p = 0.12).

C. Rates of disappearance from the blood (values of C). The series of decreasing persistence is as follows. Values of C are in hours.

Sulphamerizine 7.4 ± 0.95; sulphadiazine 6.5 ± 0.5 (d); sulphamezathine 4.2

± 0.65; sulphapyridine 4.15 ± 1.2 (d); sulphaguanidine 2.2 ± 0.85; sulphanilamide 1.95 ± 0.75 (s); sulphathiazole 1.0 ± 0.35.

The difference between sulphamerizine and sulphadiazine is not quite significant ($p = 0.055$).

Discussion. Data on the absorption of all of these drugs in the mouse have previously appeared (3, 4, 5, 6, 7, 8, 9, 10, 11) but they had not always been examined by a standard technique nor in sufficient detail. Comparison of the present results with those of other workers shows certain differences, particularly in the magnitude of the blood concentrations recorded (cf. 11). In general, however, earlier findings are confirmed, particularly as regards the marked superiority in absorption properties of the three sulphapyrimidines. All give high blood concentrations and are very persistent, and sulphamezathine is rapidly absorbed. Among other drugs the poor absorption of sulphaguanidine and the poor persistence of sulphathiazole are noteworthy.

TABLE 7

Blood levels of sulphadiazine in the mouse

EXPT. NO.	BLOOD LEVELS IN MGM./100 ML. AFTER									MAX.	T MAX.	C7
	20 min.	40 min.	1 hr.	1½ hr.	2½ hr.	3½ hr.	5 hr.	7 hr.	24 hr.			
										mgm./100 ml.	min.	hr.
1	10.5	11.1	14.3	11.6	14.8	12.7		15.2	1.5	14.0	240	7.7
2	9.6	11.6	11.3	11.8	12.4	15.4	13.8	13.3	1.2	14.0	250	6.6
3	8.8	10.8	14.3	15.2	13.4	15.7	14.3	17.3	4.3	16.1	160	7.3
4	12.0	14.0	16.0	14.6	15.6	13.9	13.1	11.4	1.7	16.4	100	4.7
5	12.6	11.0	16.1	17.9	20.8	18.0	14.8	13.9	0	18.8	150	4.7
6	12.6	14.1	15.8	20.5	19.5	19.2	15.3	12.9	1.3	19.6	170	5.1
7	13.2	15.4	17.8	18.0	14.0	12.4	9.7	8.3	2.2	18.0	80	7.2
8	15.5	18.2	19.6	21.5	19.6	17.7	14.6	13.8	3.4	20.0	100	6.6
9	16.0	15.1	17.3	20.3	19.2	23.5	16.7	18.8	1.4	19.2	150	5.9
10	9.8	13.4	15.5	16.0	13.4	21.0	18.4	13.5	2.5	16.8	180	6.3
11	13.1	19.9	30.0	20.5	24.2	16.2	23.0	12.8	2.9	20.8	120	6.6
12	10.0	12.6	15.0	9.6	22.0	19.0	21.0	19.6	3.5	19.2	240	7.0
13	7.8	14.6	20.0	31.0	16.3	22.7	17.8	18.8	3.5	19.4	230	7.2
14	13.8	15.6	7.3	13.9	20.6	15.6	14.3	17.4	1.5	16.6	120	5.8
15	16.0	18.4	15.7	14.4	17.0	18.3	19.0	15.6	3.3	17.7	180	7.3
Means	12.1	14.4	16.4	17.2	17.5	17.4	16.1	14.8	2.3	17.8	165	6.4
Standard deviation										2.1	55.6	1.0
Standard error of mean										0.55	14.4	0.3

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THE ANTIMALARIAL ACTION IN DUCKS OF CERTAIN SULFANILAMIDE DERIVATIVES[1]

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In a previous publication (1) it was shown that various sulfanilamides are very effective in suppressing parasitemia in lophurae malaria of ducks; that optimal therapy depends upon the maintenance of more or less constant blood concentrations of these drugs; and, finally, that p-aminobenzoic acid has an antagonistic effect on sulfanilamides in *P. lophurae* as has been reported by Seeler and his associates (2) and by Maier and Riley (3) for *P. gallinaceum*. The complete inactivity, reported by other investigators, of sulfanilamides against relictum and cathemerium malaria in the canary was considered by us as possibly due not only to species differences in parasite susceptibility but also to the use of single daily doses of these drugs instead of the maintenance of continuous blood concentrations.

In the present communication data are presented to show 1) that the maximum reduction of parasitemia that can be obtained on the sixth day with the sulfanilamides is a reduction from 70 per cent to 1 to 2 per cent cells parasitized and elevation of drug dosage beyond this point yields no further drop in parasitemia,[2] 2) that the sulfanilamides act much more slowly than quinine in lophurae malaria in ducks, 3) that the sulfanilamides in general clinical use are much more active in lophurae than in cathemerium malaria in the duck, and 4) that certain halogen substituted sulfanilanilides appear to differ from other sulfanilamides in their mode of action.

Methods. These were in the main similar to those described in our previous communication (1). For the lophurae infection, the dose of infected blood was 100×10^6 parasitized cells per bird, giving a peak of parasitemia on the sixth day, except that in certain experiments the dose was decreased to give a peak on the tenth day. For the cathemerium infection a dose of 50×10^6 parasitized cells per bird was used and this gave a peak on the fourth or fifth day.[3] White Pekin ducklings, two weeks old, and weighing about 100 grams were used in all experiments. The drug-diet method with a six-hour light-dark cycle was used for treatment. In all cases drug-diet treatment was started twenty-four hours before infection. Drug activity was measured in terms of reduction in per cent of cells parasitized (% C.P.) at a time when infection in untreated birds was at or near the peak. In counting parasitized cells, the number of red blood cells examined varied inversely with the degree of parasitemia between the limits of 200 cells for 1% C P and greater and 10,000 cells for 0 01% C P

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[1] This investigation was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University. The experiments described in this paper were completed early in 1943. Security reasons first and later pressure of important war work prevented their preparation for publication.

[2] Since the experiments described in this paper were completed, further experience with sulfadiazine in lophurae malaria of the duck has indicated that a decrease of infecting dose from 100 to 50×10^6 parasitized cells may decrease parasitemia on the sixth day from 1 to 2 per cent to less than 0.5 per cent cells parasitized. This would tend to indicate that the dosage of infecting parasites may have an effect on the degree of action of sulfadiazine. Under the conditions of our experiments sulfadiazine acts more slowly than quinine and unlike quinine fails to reduce the parasitemia to a very low value on the sixth day.

[3] We wish to thank Dr. Fruma Wolfson for furnishing us with *P. cathemerium* (Strain 3T) which had been repeatedly passed in ducks.

TABLE 1

Comparison of speed and degree of antimalarial action of sulfadiazine and quinine

DRUG	GM DRUG PER 100 GM DIET	ESTIMATED DRUG INTAKE	BLOOD CONC	GEOMETRIC MEAN OF % C. P. ON DAY						NUMBER DUCKS
				1	2	3	4	5	6	
		mg/kg/day	mg %							
Sulfadiazine	2/10	800	6.6	0.9	4	8	7	4	3	3
	1/10	400	4.4	0.7	4	8	7	6	1	10
	1/20	200	1.9	0.9	6	9	8	6	2	10
	1/40	100	1.1	0.9	4	9	9	5	2	10
	1/80	50	0.8	0.7	5	8	9	8	3	10
	1/160	25		0.9	6	8	8		7	10
Quinine	1/20	160		0.6	0.3	0.1	0.05	0.04	0.02	5
	1/40	80		0.8	0.7	0.7	0.5	0.2	0.05	5
	1/80	40		0.7	3	8	15	20	20	5
Controls				1	5	7	30	50	70	12

Infection: *P. lophurae*, 100 × 10^6 parasitized cells per duck.
Duration of drug-diet therapy: 6 days.
% C.P. = Per cent of Red Blood Cells Parasitized.

TABLE 2

Proportion of altered parasites

SULFADIAZINE GM PER 100 GM DIET	BLOOD CONC	GEOMETRIC MEAN OF % C P ON DAY		PERCENTAGE*			NUMBER DUCKS
		6	7	G	A	AA	
	mg %						
8/10	21	4	2	22	15	63	8
1/10	3.4	2	1	31	5	64	7
1/80	0.5	4	3	29	51	20	7
Controls		70	80				5

Infection. *P lophurae*, 100 × 10^6 parasitized cells per duck.
Duration of Therapy 6 days
* Percentage of kind of parasite present on 7th day
G = Gametocytes
A = Asexual forms of normal appearance
AA = Asexual forms of abnormal appearance (vacuolization or complete lack of differential staining).

Since the degree of parasitization of different individuals within a group of birds sometimes varies widely, the geometric mean of the individual values rather than their arithmetic mean was used as a measure of the degree of parasitization of the group. The use of the geometric mean to represent the response of a group of parasitized ducks to an antimalarial drug has been justified by an analysis of the individual responses to three different doses of quinine of three groups of parasitized ducks containing more than 250 members in each group. In each of these three groups it was found that a normal distribution curve was obtained when the percentage of cells parasitized in the different members of the group was plotted on a logarithmic scale. When plotted on an arithmetic scale the same data yielded a skewed distribution.

TABLE 3

Correlation between dosage and parasitemia on 5th day after end of therapy

SULFADIAZINE GM. PER 100 GM. DIET	BLOOD CONC.	GEOMETRIC MEAN OF % C.P. ON DAY 6	GEOMETRIC MEAN OF % C.P. ON DAY 11	NUMBER DUCKS
	mg.%			
8/10	17.2	1	0.03	3
2/10	8.4	2	0.2	3
1/40	0.9	5	1	3
1/80	0.4	3	10	3
Controls		60		3

Infection: *P. lophurae*, 100 × 10^6 parasitized cells per duck.
Duration of Therapy: 6 days.

TABLE 4

Viability of parasitized cells after 6 days exposure to sulfadiazine in vivo

DUCK NO.	SULFADIAZINE GM. PER 100 GM. DIET	BLOOD CONC.	% C.P. ON DAY 6	% C.P. ON DAY 7		% C.P. IN SUBINOCULATED DUCKS ON DAY 6	% C.P. IN SUBINOCULATED DUCKS ON DAY 10	% C.P. IN SUBINOCULATED DUCKS ON DAY 14	% C.P. IN SUBINOCULATED DUCKS ON DAY 17
		mg.%							
33 W	8/10	24	2	1	Subinoculation from each duck on 7th day —100 × 10^6 P.C.	0.05	0.04	10	Dead
33 B	8/10	20	4	2		0.5	0.8	10	90
31 B	1/10	3.4	0.9	1		2	60	Dead	
31 Y	1/10	3.4	1	2		2	80	Dead	
32 W	1/80	0.4	3	2		10	Dead		
32 B	1/80	0.4	3	1		10	90	Dead	
29 B	Control		70	80*		60	Dead		
29 Y	Control		70	80*		80	Dead		

Infection: *P. lophurae*, 100 × 10^6 parasitized cells per duck.
Duration of Therapy: 6 days.
* 20 mg. % of sulfadiazine added at time of subinoculation.

In the present series of experiments, quinine was used as a reference standard and activity was evaluated in terms of the minimal amount of drug required to produce a significant reduction in parasitemia. The ratio of minimal effective dose of quinine to minimal effective dose of test drug was used to express relative activity, and is referred to as the quinine equivalent (4).

RESULTS. The characteristic failure of sulfanilamide derivatives to reduce the parasitemia below 1–2% C.P., regardless of dosage, when an infection of 100 × 10^6 parasitized cells with a sixth day peak is used, is illustrated for sulfadiazine in tables 1 and 2. This is in sharp contrast to the effect obtained with quinine (table 1).

Since the parasites in sulfadiazine treated birds appeared to be abnormal, the percentage of altered parasites at three dosage levels was determined (table 2). Any parasite which showed vacuolization or complete loss of differential staining was considered to be abnormal. It is evident that morphological alteration of parasites is more frequent with high doses than with the low dose.

In order to investigate the possibility that the altered parasites were non-viable, counts were made at the end of treatment on the sixth day and again on the eleventh day. These results (table 3), showing on the eleventh day, a graded effect with dosage, suggest that the altered forms present on the sixth day were non-viable. Direct evidence indicating non-viability of altered parasites was obtained in the experiment summarized in table 4. In this experiment, the infections resulting from subinoculations made on the seventh day indicate that the viability (infectivity) of the residual parasites is inversely related to the drug concentration to which the parasites had been exposed during the six-day treatment period.

TABLE 5

Antimalarial action of sulfadiazine and quinine on a slowly developing infection

DRUG	GM. DRUG PER 100 GM. DIET	GEOMETRIC MEAN OF % C.P. ON 10TH DAY	NUMBER OF DUCKS
Sulfadiazine	1/40	0.01	5
	1/80	0.01	4
	1/160	1	5
	1/320	15	5
	1/640	40	5
	1/1280	80	4
Quinine	1/40	0.01	5
	1/80	0.2	5
	1/160	70	5
	1/320	70	4
	1/640	70	5

Infection: *P. lophurae*, 1×10^6 parasitized cells per duck.
Duration of Therapy: 10 days.

When the infecting dose of parasites is reduced so that the peak of parasitemia occurs on the tenth day (table 5) sulfadiazine reduces the parasitemia below the 1 to 2 per cent limit observed with the sixth day peak infection and a graded effect is obtained, as with quinine.

It is evident from table 1 that the failure to obtain graded responses with high doses of sulfadiazine is not due to lack of absorption since the blood concentrations obtained are more or less proportional to dose. In addition, table 1 shows that the action of sulfadiazine is much slower than that of quinine as judged by the effect on parasitemia. Thus, quinine in effective dosage arrested multiplication of parasites rapidly since the number of parasitized cells declined from the first day on, while sulfadiazine in all doses permitted multiplication at the same rate as the controls through the first three days. Since *P. lophurae* has about a 36-hour cycle (5), the first new generation of parasites, 36–72 hours after the inoculation of the birds, must have been essentially normal, but development of the succeeding generation, 72–108 hours after inoculation, was impaired as shown by the fourth and fifth day counts.

The finding by Wolfson (6) that satisfactory experimental infections with *P. cathemerium* could be produced in the duck made possible the comparison of the action of the same drug in the same host on two species of avian malaria. Under these conditions any difference in activity can be ascribed to species difference in susceptibility of parasites. Table 6 summarized the relative activities of sulfanilamides against the two species of parasites.[4] It is evident from the quinine equivalents that sulfanilamides in clinical use have activity of the order of quinine in lophurae but relatively little activity in cathemerium malaria.

TABLE 6

Quinine equivalents of sulfanilamides in malaria in the duck

DRUG	QUININE EQUIVALENT	
	Lophurae	Cathemerium
Sulfadiazine	2	1/32
Sulamerazine	1	<1/8
Sulfapyrazine	4	<1/8
Sulfaguanidine	1/2	<1/16
3',5'-Dibromosulfanilanilide	1/4	1/2
3',5'-Dichlorosulfanilanilide	1/4	1/2

Infection· *P. lophurae*, 100 × 10^6 parasitized cells per duck.
Duration of Therapy: 6 days.
Infection *P. cathemerium*, 50 × 10^6 parasitized cells per duck.
Duration of Therapy: 5 days.

Of considerable interest is the finding that two halogenated sulfanilanilides are fully as active on cathemerium as on lophurae malaria. In view of these findings which suggest a mode of action different from that of the ordinary sulfanilamides, the antagonistic action of p-aminobenzoic acid on these compounds and on sulfadiazine was compared. These experiments are summarized in table 7. The activity of the halogenated sulfanilamides was not significantly affected by an amount of p-aminobenzoic acid which completely antagonized sulfadiazine.

DISCUSSION. The data presented in this communication supplement and extend the observations made previously (1). It has been found that clinically used sulfanilamides are as active as quinine on the basis of minimal dosage giving significant reduction of parasitemia. However, these compounds failed to reduce parasitemia below a level of 1 to 2% C.P. (with a sixth day peak infection in the controls). The data given in tables 1–5 to a large extent explain this failure by showing that: 1) the residual parasites on the sixth day are abnormal in appearance, 2) the proportion of abnormal parasites correlates to some extent with dosage, 3) the altered parasites are apparently non-viable since they disappear between the end of treatment on the sixth day and the eleventh day with the result that a graded effect from dosage becomes evident, and 4) the viable count on the sixth day, determined by subinoculation experiments, correlates with the dosage.

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[4] The sulfadiazine was supplied by the American Cyanamid Company, the sulfamerazine by Sharp and Dohme; the sulfapyrazine by Mead Johnson and Company, and the sulfaguanidine by E R Squibb and Sons The bromosulfanilanilide was obtained partly from the Winthrop Chemical Company, Inc and partly from Joseph Koepfli. The chlorosulfanilanilide was prepared in this laboratory by R G Shepard.

The slow action of sulfadiazine is most apparent when parasitemia-time curves for this drug are compared with those obtained for quinine. Furthermore, the curve for sulfadiazine appears to be identical with that of the control birds until after the third day. It is probable that the slow action of the sulfanilamides is partially responsible for their failure to lower parasitemia below 1 to 2% C.P. in an infection of 100×10^6 parasitized cells per bird which gives a sixth day peak in the control birds. This interpretation is substantiated by the observation that a fully graded dosage-effect relationship was obtained similar to that with quinine when the action of sulfadiazine was investigated on a slowly developing infection (tenth day peak).

TABLE 7

Antagonistic action of p-aminobenzoic acid on sulfanilamides

DRUG	GM. PER 100 GM. DIET	p-AMINOBENZOIC ACID GM. PER 100 GM. DIET	GEOMETRIC MEAN OF % C.P. ON 6TH DAY
3′,5′-Dibromosulfanilanilide	1/10	0	0.2
3′,5′-Dibromosulfanilanilide	1/20	0	15
3′,5′-Dibromosulfanilanilide	1/10	1/10	0.4
3′,5′-Dibromosulfanilanilide	1/20	1/10	40
Sulfadiazine	1/10	0	2
Sulfadiazine	1/10	1/10	60
Controls			70
3′,5′-Dichlorosulfanilanilide	1/10	0	0.3
3′,5′-Dichlorosulfanilanilide	1/10	1/10	0.5
3′,5′-Dichlorosulfanilanilide	0	1/10	60
Controls			60

Infection: *P. lophurae*, 100×10^6 parasitized cells per duck.
Duration of Therapy, 6 days.

The comparatively slow action of sulfanilamides in gallinaceum malaria has been studied by Brackett, Waletzky and Baker (7). They obtained definite evidence that the multiplication of parasites was inhibited as early as 24 to 36 hours after exposure to sulfadiazine. Our data for lophurae malaria do not show inhibition of parasites before 72 hours exposure to sulfadiazine. It is possible that this slight discrepancy reflects a difference in species susceptibility.

It should be noted that the same proportional increase in dosage does not produce the same reduction in parasitemia in the case of sulfanilamides as in the case of quinine. This difference makes it difficult to compare the effectiveness of the sulfanilamides with that of quinine. For example in table 1, sulfadiazine appears to be 2 to 4 times as active on the basis of minimal dose necessary to produce significant reduction in parasitemia, yet on the basis of the ability to suppress parasitemia quinine is obviously superior. In addition to the difficulty encountered in the case of the sulfanilamides, it should be noted that *only* on the basis of minimal effective doses can slightly active drugs be compared to quinine. Therefore, it would appear to be advisable to use this basis for the purpose of evaluating the relative anti-malarial activity of drugs in general.

The experiments comparing relative activity of drugs on two species of malarial parasites in the same host indicate real differences in species susceptibility since host differences in susceptibility and drug metabolism are eliminated.

The two halogenated sulfanilanilides studied were reported to be active on relictum malaria in the canary (8). The evidence presented here with regard to these drugs clearly indicates that their mode of action in experimental malaria is different from that of the usual sulfanilamides in that 1) they are highly active against cathemerium malaria, and 2) their action is not antagonized by p-aminobenzoic acid.

SUMMARY

1. Sulfanilamide derivatives such as sulfadiazine possess high activity compared to quinine in lophurae malaria in ducks when the comparison is based on minimal effective doses. However, these derivatives fail to reduce the parasitemia below 1 to 2% cells parasitized on the sixth day regardless of dosage when tested against an infection having a sixth day peak parasitemia in the controls.
2. The antimalarial action of sulfanilamides, such as sulfadiazine, is slower than that of quinine.
3. 3′,5′ Dibromosulfanilanilide and 3′,5′ dichlorosulfanilanilide exhibit a mode of action which differs qualitatively from that of ordinary sulfanilamides.
4. It is advisable to use minimal effective doses as the basis of comparisons of antimalarial activity.

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ACUTE TOXICITY OF VASOPRESSOR AMINES

I. EFFECT OF TEMPERATURE

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The results of numerous investigators (1, 2, 3, 4, 5) demonstrate that the toxicity of a wide variety of chemical substances is markedly affected by fluctuations in environmental temperature. Pfeiffer and co-workers (4) reported the toxicity of Amphetamine and Neo-Synephrine was decreased by lowered environmental temperature while that of Ephedrine was unchanged. Leser and co-workers (6) showed artificial cooling of mice resulted in increased mortality after injections of Amphetamine.

Incidental observations made in this laboratory (7) indicate that the subcutaneous LD_{50} of Ephedrine in rats is decreased by at least 100 per cent when animals are placed in a chamber through which warm air is circulated. The toxicity of certain other vasopressor amines was either unaltered or increased to a lesser extent under similar conditions. Several factors might have been responsible for the observed alteration in toxicity; however, it appeared likely that temperature was the most important. Thus, the present study was undertaken to investigate effects of environmental temperature on the toxicity of various vasopressor amines.

This investigation includes the determination of LD_{50} values for these compounds in mice at 26 and 32°C. following subcutaneous and intravenous injections and studies on rectal temperature and mortality following subcutaneous injections of Ephedrine and Vonedrine in rats.

METHODS. All animals employed in this investigation were housed at approximately 26°C. Those subjected to 32°C. were conditioned to this temperature for two hours before drug administration.

The white mice weighed 16 to 19 grams. Both subcutaneous and intravenous doses were administered in a total volume of 0.25 cc. in this species. LD_{50} values and standard errors were obtained by the method of Miller and Tainter (8).

Young male white rats weighing 180 to 240 grams or older rats weighing 300 to 500 grams were employed in studies on rectal temperature. The animals were injected subcutaneously with fractional parts of the LD_{50} as determined at 26°C. Rectal temperatures were taken prior to injection and at hourly intervals for six hours thereafter.

The hydrochlorides of the following amines were studied: Ephedrine (*l*-1-phenyl-2-methylaminopropanol-1); Propadrine (*dl*-1-phenyl-2-aminopropanol-1); Amphetamine (*dl*-1-phenyl-2-aminopropane); Tuamine (*dl*-2-aminoheptane); Vonedrine (*dl*-1-methylamino-2-phenylpropane); and Privine (2-[α-naphthylmethyl] imidazoline). All solutions were made with pyrogen-free, sterile, distilled water.

RESULTS. Data presented in table 1 show that the acute subcutaneous toxicities in mice of Ephedrine, Amphetamine, and Propadrine are markedly increased by an elevation of the environmental temperature while those of Tuamine, Vonedrine, and Privine are not significantly altered.

The data in table 2 show that the toxicity of Ephedrine is increased while that of Vonedrine is unaltered by increased environmental temperature following intravenous injections. This indicates that the observed difference in effect of environmental temperature on the subcutaneous toxicity of the two groups of compounds is not related to differences in absorption.

It was noted following intravenous administration that all deaths with Vonedrine at both temperatures and Ephedrine at 26°C. occurred within three minutes after injection; however, some doses of Ephedrine proved lethal five hours after administration at 32°C. This increase in the period of mortality probably is related to the more persistent temperature-raising action of Ephedrine. This effect of Ephedrine as well as of Amphetamine, Pervitin, and Veritol on body temperature has been demonstrated by Kiessig (9). Differences between Ephedrine and Vonedrine with respect to this action are apparent from data obtained in rectal temperature studies.

TABLE 1

LD_{50} values in mgm./kgm. for subcutaneous administration in mice subsequently kept at 26° and 32°C.

COMPOUND (HYDROCHLORIDE)	LD_{50} AT 26° C.	LD_{50} AT 32°C.
Ephedrine	600 ± 54.8	83 ± 8.9
Propadrine	600 ± 49.0	214 ± 26.3
Amphetamine	42 ± 4.2	15 ± 1.6
Tuamine	115 ± 11.6	76 ± 6.5
Vonedrine	540 ± 48.0	400 ± 37.0
Privine	170 ± 13.8	150 ± 11.0

TABLE 2

LD_{50} values in mgm./kgm. obtained at 26° and 32°C. following intravenous injection in mice

COMPOUND (HYDROCHLORIDE)	LD_{50} AT 26°C.	LD_{50} AT 32°C.
Ephedrine	100 ± 6.3	36 ± 4.1
Vonedrine	60 ± 3.4	65 ± 7.2

Table 3 shows the effect of varying doses of these two compounds on the rectal temperature of rats (300 to 500 grams) at 26°C. None of the doses of Ephedrine and Vonedrine caused an increase in rectal temperature amounting to 5°F. or more. None of the experimental animals died, and the single difference between the two compounds was that temperature rises persisted longer following the administration of Ephedrine than they did following Vonedrine.

Studies carried out with rats (300 to 500 grams) kept at 32°C. indicated no significant differences between Ephedrine and Vonedrine with reference to effects on rectal temperature. Generally, when the temperature rose over 5°F. and was maintained for any considerable period, death followed with either compound. Some difference between the amines was noted with respect to mortality. Five per cent of the LD_{50} (determined at 26°C.) caused four deaths out of six animals which received Ephedrine, while 5 per cent of the LD_{50} of Vonedrine caused two deaths out of six animals. Twenty per cent of the ordinary LD_{50} proved fatal to all six animals in each series.

Table 4 shows that Ephedrine caused a greater temperature rise and higher mortality rate than did Vonedrine in young rats (180 to 240 grams) at 32°C.

TABLE 3

The effect of subcutaneous administration of fractional parts of the LD_{50} of Ephedrine and Vonedrine on rectal temperatures of rats (300 to 500 grams) kept at 26°C.

COMPOUND (HYDROCHLORIDE)	DOSE	LD_{50}	RATS	TEMPERATURE INCREASE	AVERAGE DURATION OF MAXIMAL TEMPERATURE
	mgm./kgm.	*per cent*	*number*	*°F.*	*hours*
Ephedrine	15	5	6	2.2 to 4.4	<1
	60	20	6	1.0 to 4.6	4
Vonedrine	42.5	5	6	0.0 to 2.0	<1
	170.0	20	6	1.4 to 4.0	1

TABLE 4

The effect of subcutaneous administration of fractional parts of LD_{50} of Ephedrine and Vonedrine on rectal temperature and mortality of rats (180 to 240 grams) kept at 32°C.

COMPOUND (HYDROCHLORIDE)	DOSE	LD_{50}*	RATS	TEMPERATURE INCREASE	MORTALITY
	mgm./kgm.	*per cent*	*number*	*°F.*	*per cent*
Ephedrine	15	5	6	3.7 to 7.0	67
	60	20	6	3.8 to 8.8	83
Vonedrine	42.5	5	6	0.4 to 3.2	0
	170.0	20	6	1.4 to 6.6	33

*Values determined in 300 to 500 gram rats at 26°C.

Data on durations of effect on temperature are not included in this table because of inadequate numbers of surviving animals. However, there were clear indications that in this experiment, also, Ephedrine had a more persistent effect.

These results demonstrate that the differences between Vonedrine and Ephedrine and likely other compounds in the two groups must be regarded as quantitative rather than qualitative ones.

Discussion. Several factors may be responsible for the unaltered toxicity of Vonedrine in mice, some increase in toxicity of this compound in young rats, and the greater increase in toxicity in older rats at the increased environmental temperatures. One important factor likely is that of differences in the regions of thermal neutrality. Herrington (10) has shown that rectal temperatures of mice are comparatively stable in environmental temperatures up to 33°C., and those of older rats (310 to 377 grams) remain rather constant up to 29°C. At environmental temperatures above these, rectal temperature increases rapidly for each small increase in environmental temperature. Since Vonedrine has a comparatively mild effect on body temperature its toxicity at 32°C. is not increased in the mouse, a species with stable body temperature at a fairly high environmental temperature. Its toxicity is increased, however, in heavy rats because these animals have a comparatively low region of thermal stability. The mild temperature-raising effect of Vonedrine is sufficient to upset their temperature regulation to a serious degree. The toxicity of Ephedrine, on the other hand, is increased by elevated environmental temperature in both species. This is evidently due to the greater and more persistent temperature-raising action of Ephedrine which must be sufficiently intense to upset seriously temperature regulation even in the mouse at the elevated temperature.

SUMMARY

The acute subcutaneous toxicities in mice of Ephedrine, Propadrine, and Amphetamine were increased by the elevation of environmental temperature while those of Tuamine, Vonedrine, and Privine were not significantly altered. Intravenous administrations indicate these effects were not due to alterations in rates of absorption.

Ephedrine had a greater and more persistent temperature-raising action than Vonedrine following subcutaneous injection in rats, and Ephedrine toxicity was increased more than that of Vonedrine in this species. Thus, altered toxicity at different temperatures appears to be correlated with effects of the amines on body temperature.

Effects of environmental temperature on the toxicity of various vasopressor amines were modified by weight (age) of animals and the species employed.

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ACUTE TOXICITY OF VASOPRESSOR AMINES

II. Comparative Data

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It has been shown previously (1) that fluctuations in environmental temperature and age (weight) of animals alter the toxicity of certain vasopressor amines. Chen and Robbins (2, 3, 4) have recently reported on the effect of age on the toxicity of various drugs. They found that rats one to one and one-half months old are more susceptible to Ephedrine hydrochloride injected intravenously than younger or older ones; and their results indicate, in general, that one cannot predict effects of age of animals on drug action since each substance has its own characteristics. These factors of environmental temperature, age and weight of animals and others, such as strain of animals, place of injection, and rate of injection, probably account for the marked variation in LD_{50} values which have been reported for various vasopressor amines.

In view of the extensive clinical uses of these therapeutic agents and the inconsistent toxicity data reported in the literature, it seemed desirable to compare their toxicities following several modes of administration in different species of animals. The investigation reported here was done over a relatively short period of time, and various factors which might alter toxicity were reduced to a minimum. Therefore, the data obtained should define comparative acute toxicity of the chemical substances investigated with fair accuracy.

METHODS. A total of 610 New Zealand rabbits from the same rabbitry, 839 white rats of the Wistar strain, and 222 white mice of the Swiss strain were employed in determining LD_{50} values. The rabbits weighed between 2 and 3 kilograms and the mice between 16 and 20 grams. Rats of two different weight groups were employed. One group included young mature animals which weighed between 190 and 250 grams and the other older and heavier animals which weighed 300 to 500 grams.

Intravenous injections in rabbits were made into the marginal ear vein. Concentrations employed were such that one LD_{50} was administered every one to two minutes. Intramuscular injections in rabbits were made into the thigh muscles. Subcutaneous injections in all species were made in the mid-dorsal region. Intraperitoneal injections in rats were made in such a manner as to prevent injection into internal organs.

The animals were quartered in a room having a temperature of 26°C. with variations of one degree plus or minus. All deaths occurring within two weeks after drug administration were included in estimating acute toxicity values.

The hydrochloride salts of the following amines were studied: Vonedrine (*dl*-1-methylamino-2-phenylpropane); Ephedrine (*l*-1-phenyl-2-methylamino-propanol-1); Propadrine (*dl*-1-phenyl-2-aminopropanol-1); Tuamine (*dl*-2-aminoheptane); Amphetamine (*dl*-1-phenyl-2-aminopropane); Privine (2-[α-naphthylmethyl] imidazoline); and Neo-Synephrine (*l*-1-[m-hydroxyphenyl]-2-methylaminoethanol).

RESULTS. When the LD_{50} is used as the criterion of toxicity, it will be noted (table 1) that Vonedrine, Ephedrine, and Propadrine are the least toxic compounds and are of the same general order of toxicity. One significant difference between LD_{50} values in this group is noted following subcutaneous injection in heavy rats (300 to 500 grams). Vonedrine toxicity is not altered in heavier rats while that of Ephedrine and Propadrine is increased. This may be due to the temperature-raising action of Ephedrine and Propadrine and an increased susceptibility of older rats to this effect (1). Another marked difference between Vonedrine and Ephedrine is in the slope of mortality curves for the subcutaneous route in rats. The curves for Vonedrine are steep while those for Ephedrine are steep in the higher dosage range but flatten out in the lower dosage levels. For example, Ephedrine injected subcutaneously proved lethal to young rats in doses ranging from 180 to 950 mgm./kgm. while Vonedrine proved lethal in the narrow dosage range of 750 to 950 mgm./kgm. This factor, and not small groups of test animals, accounts for the large standard errors of certain determinations.

TABLE 1

LD_{50} values in mgm./kgm. with standard errors obtained by method of Miller Tainter (6)

COMPOUND (HYDROCHLORIDE)	INTRAVENOUS	INTRAPERITONEAL	INTRAMUSCULAR	SUBCUTANEOUS			
	Rabbits	Heavy rats	Rabbits	Rabbits	Light rats	Heavy rats	Mice
Vonedrine	72 ± 1.7	165 ± 9.2	220 ± 15.0	205 ± 15.9	850 ± 36.7	850 ± 34.3	540 ± 48.0
Ephedrine	60 ± 3.2	165 ± 15.2	175 ± 9.2	165 ± 12.3	650 ± 109.4	320 ± 40.6	600 ± 54.8
Propadrine	50 ± 3.1	160 ± 6.7	320 ± 29 2	255 ± 16.3	660 ± 51.6	380 ± 27.1	600 ± 49.0
Tuamine	22 ± 1.4	34 ± 2.2	85 ± 5.7	130 ± 14.4	135 ± 6.1	160 ± 13.0	115 ± 11.6
Amphetamine	10 ± 1 6	30 ± 3.0	10 ± 0 8	11 ± 1.2	165 ± 16.5	39 ± 2.3	42 ± 4.2
Privine ..	0 8 ± 0.09	50 ± 3.6	0.95 ± 0.23	0.95 ± 0.15	385 ± 39.6	325 ± 22.5	170 ± 13 8
Neo-Synephrine	0.5 ± 0.15	17 ± 1.1	7.2 ± 0.35	22 ± 2.2	27 ± 2.9	33 ± 2.0	22 ± 4.3

Tuamine, Amphetamine, Privine, and Neo-Synephrine vary considerably in toxicity with various types of administration in the species employed; however, they are the more toxic group. Privine and Neo-Synephrine are most toxic intravenously in rabbits. Privine also has a high order of toxicity in this species following subcutaneous and intramuscular injection, but its toxicity following subcutaneous injection in rats and mice is low when compared to that for other members of this group.

SUMMARY

Comparative LD_{50} values are presented for seven vasopressor amines.

Vonedrine, Ephedrine, and Propadrine have a similar order of toxicity and constitute the low toxicity group. Vonedrine differs from the other two amines in this group in that it is less toxic following subcutaneous injection in older and heavier rats.

Tuamine, Amphetamine, Privine, and Neo-Synephrine are the most toxic of

the amines. The two latter compounds have a comparatively high order of toxicity following intravenous injection in rabbits; and Privine is especially toxic for the rabbit following all modes of administration investigated. This compound, however, is the least toxic of the four in this group following subcutaneous injection in rats and mice.

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RELATION OF ABSORBABILITY TO THE COMPARATIVE TOXICITY OF DDT FOR INSECTS AND MAMMALS

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Since DDT has gained prominence as an insecticide, its toxicity for insects has most frequently been expressed either in terms of percent kill after exposure to a given environmental concentration of the agent (dispersed in air on water or on surfaces, etc.) for a given length of time, or in terms of efficiency of eradication of an insect pest from a given area (1). Such data are invaluable from the point of view of field application, but studies on mechanism of action require knowing the lethal dose per insect. It is standard procedure to express such a dose as the LD-50, viz. the dose required to kill 50 per cent of a suitably large group of animals. An early estimate, not based on actual measurement, suggested that flies are killed by as little as 10^{-5}–10^{-6} micrograms per fly (2). Assuming a fly weight of 20 mgm. this latter figure translates to 0.05 micrograms per kgm. Such an order of toxicity would be remarkable indeed. Some direct measurements have been made of doses required for kill. Metcalf and Kearns administered measured doses of DDT to the cockroach (*Periplaneta americana*) (3). They reported a lethal dose of the order of 30 mgm. of DDT per kgm., applied to the body surface in emulsion form. David has made measurements of the quantity of DDT collected from a spray by flying insects. His figures for "maximum median lethal dose" are 7 and 10 mgm. per kgm. for the male and female of *Musca domestica*, and 5 and 7 mgm. per kgm. for the male and female of *Aedes aegypti* (4). Latta, Anderson and Rogers reported that the approximate amount of surface deposited DDT in oil necessary to kill an *Aedes aegypti* female of average susceptibility is about 0.03 gamma per mosquito (5). Assuming an average body weight of 1–3 mgm. per mosquito this datum calculates to 10–30 mgm. per kgm.

Studies on mammals indicate that the acute LD-50 of emulsified DDT, injected intravenously, is 40–50, 35–50, 25–40, 60–75 and 50–60 mgm. per kgm. of body weight for the rat, rabbit, cat, dog and monkey respectively (6). On the other hand, DDT powder applied to the rabbit skin, dry or wet with physiological saline, is non-toxic in doses as high as 4 grams per kgm. Applied to the skin in certain organic solvents, however, it is toxic (7).

Collecting these data then, one sees that the average LD-50 of surface applied DDT for the cockroach, fly and mosquito is about 12 mgm. per kgm., that of intravenously injected, emulsified DDT in the mammal is about 49 mgm. per kgm., and that of dry DDT applied to the rabbit skin is very high (over 4 grams per kgm.). Other studies also indicate that dry DDT is relatively non-toxic for mammals when applied to the skin (8).

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[1] This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

The data thus suggest that the factor which makes DDT appear to be so much more toxic for insects than mammals is one of absorbability. The importance of the absorbing surface is further indicated by the finding of a positive correlation between the possession of a chitinous exoskeleton and susceptibility to DDT (9).

To further substantiate this conclusion and to obtain additional, directly measured toxicity data for insects we have determined the approximate LD-50 for DDT injected into the abdomen of the cockroach (*Periplaneta americana*) in various solvents, and applied to the surface of the cockroach and the fly (*Musca domestica* and *Calliphora*) in acetone.

METHODS. In all experiments, the DDT solution was delivered through a 27 gauge hypodermic needle from a 0.25 cc. tuberculin syringe fitted with a hand driven micrometer screw for advancing the piston (10). Such an instrument properly calibrated, can be used to deliver volumes of the order of 1 cmm. For injection, the unanesthetized roach was held by the wings, with the ventral abdominal surface up. Since injection through the sclerotized cuticle may allow hemolymph to exude from the hole which remains upon withdrawal of the needle, care was taken to slip the needle under a sternite so that it entered the abdomen through thin body wall at the hinge like point of attachment. When it was withdrawn the sternite fell back into place and sealed the hole. We have rarely seen any leakage with this technique. For surface toxicity in the roach, DDT was applied in acetone solution to the dorsum of the abdomen and thorax, under the wings and near the point of attachment. Since there is little movement of the wings they cover the contaminated surface and thus help to protect against loss of DDT. The material was delivered from the device described above, but a blunted needle was used being held flat against the body surface during delivery. Such a procedure minimizes error due to evaporation of acetone solution in the needle. Since the acetone evaporates very rapidly and one then sees the dry powder left behind, we feel that this method of administration is a good approximation of the normal mode of surface contamination. Flies were lightly anesthetized with ether during surface application. Subsequent to poisoning, roaches were kept in battery jars in groups of 10

TABLE 1

Approximate LD 50 of DDT for the cockroach and fly

ANIMAL	ROUTE	SOLVENT	APPROX. LD 50, MGM./KGM.	
			At 24 hours	At 120 hours
Cockroach (*Periplaneta americana*)	Intraabdominal	Acetone		5–8
		Emulsion*		18
		Peanut oil		82
	Surface	Acetone		10
Fly (*Musca domestica*) newly emerged	Surface	Acetone	2	
Fly (*Musca domestica*) older adults	Surface	Acetone	8–21	
Fly (*Calliphora spp.*) older adults	Surface	Acetone	9–28	

* Emulsion. 1 per cent DDT, 10 per cent peanut oil, 1 per cent lecithin, 88 per cent physiological saline (6).

or fewer with water and food continuously available. Flies were kept in groups of 5 in wide-mouthed 150 cc. jars, with liquid food available.

Results. The detailed data are shown in tables 2 through 8 and are summarized (table 1) in terms of the approximate LD-50 values (graphically determined).

TABLE 2

Toxicity of DDT injected intraabdominally in acetone into cockroaches

NO. ROACHES	VOL. INJECTED	DDT	PER CENT MORTALITY AT HOURS INDICATED				
			24	48	72	96	120
	cmm.	*mgm./kgm.*					
40	1	0	0	0	0	5	5
10	2	0	0	0	20	30	40
5	4	0	0	60	80	80	80
10	8	0	10	50	70	70	70
35	1	0.1	9	11	11	11	11
45	1	1.0	7	24	24	31	33
45	1	5.0	7	24	33	33	35
45	1	10.0	16	40	51	65	65
10	1	15.0	0	10	40	50	80

Approximate 120 hour LD-50, 5–8 mgm. per kgm.

TABLE 3

Toxicity of DDT applied in acetone to the surface of cockroaches

NO. ROACHES	VOL. INJECTED	DDT	PER CENT MORTALITY AT HOURS INDICATED				
			24	48	72	96	120
	cmm.	*mgm./kgm.*					
10	1	0	0	0	0	0	0
33	1	0.1	0	3	6	15	15
8	1	1.0	0	0	13	13	13
28	1	5.0	0	7	14	18	18
31	1	10.0	0	13	37	40	52
25	1	30.0	0	28	52	85	85

Approximate 120 hour LD-50, 10 mgm. per kgm.

From table 2 it can be seen that the volume of acetone used for injection into the cockroach (1 cmm.) was itself responsible for death in 5 per cent of control animals, and a later experiment showed that 1 cmm. of intraabdominally injected acetone increases mortality from surface applied DDT. It is, therefore, reasonable to assume that the LD-50 figure of 5 mgm. per kgm. is somewhat too low and that the toxicity of DDT in acetone is about the same, *for the roach*, whether applied to the body surface or injected intraabdominally. The reduced toxicity of DDT in peanut oil is probably a function of its relatively high solubility in this viscous oil and subsequent slow release to the tissues of the animal.

Discussion. With the exception of one estimate (2), it is seen that the data of several investigators, using different insects and methods, agree rather well.

TABLE 4

Toxicity of DDT injected intraabdominally in peanut oil into cockroaches

NO. ROACHES	VOL. INJECTED	DDT	PER CENT MORTALITY AT HOURS INDICATED				
			24	48	72	96	120
	cmm.	*mgm./kgm.*					
6	2–12	0	0	0	0	0	0
30	1	10	7	13	17	20	23
30	1	48	10	13	23	27	30
29	1	95	14	31	45	48	55
30	1	200	20	40	63	63	73
6	2	200	33	33	50	50	66
6	4	380	33	50	66	83	100
6	8	760	33	83	83	83	83
9	12	1140	0	45	100		
3	20	1900	20	100			

Approximate 120 hour LD-50, 100 mgm. per kgm.

TABLE 5

Toxicity of DDT injected intraabdominally in emulsion into cockroaches*

NO. ROACHES	VOL. INJECTED	DDT	PER CENT MORTALITY AT HOURS INDICATED				
			24	48	72	96	120
	cmm.	*mgm./kgm.*					
10	1	0	0	0	0	0	0
10	1	0.1	0	20	20	20	20
10	1	1.0	0	0	0	0	0†
30	1	5.0	7	20	23	23	23
29	1	10.0	14	25	35	35	35
20	1	30.0	45	65	65	65	65
5	1	50.0	0	0	0	60	60
10	1	100.0	100				

* Emulsion: 1 per cent DDT, 10 per cent peanut oil, 1 per cent lecithin, 88 per cent physiological saline.

† Reason for this aberrant result is unknown.

Approximate 120 hour LD-50, 20 mgm. per kgm.

Interestingly enough the toxicity of DDT, with the exception of greater toxicity for newly emerged flies, is of the same order of magnitude for all the insects studied. Death, however, at doses near the LD-50, occurs much more rapidly in the fly than in the cockroach.

The data show emulsified DDT to be of about the same order of toxicity

TABLE 6

Toxicity of DDT applied in acetone to the surface of newly emerged flies (Musca domestica)

NO. FLIES	VOL. APPLIED	DDT	PER CENT MORALITY AT HOURS INDICATED				
			3	24	48	72	96
	cmm.	*mgm./kgm.**					
5	0	0	0	0	0	0	0
10	0.2	1	20	20	20	20	20
11	0.4	1.5	0	36	36		
29	0.8	2	31	45	49		
8	1.0	2.5	87	87	87		
10	0.6	3	50	100			
10	0.8	4	80	100			
5	1.0	5		100			
5	1.0	5	80	100			
5	0.4	10		100			
5	0.6	15		100			
5	0.8	21		100			
5	0.2	51	80	100			
5	0.4	103	100	100			
6	0.8	205	100	100			

* Average body weight of newly emerged flies: 3.9 mgm. (55 flies).
Approximate LD-50, 2 mgm. per kgm.

TABLE 7

Toxicity of DDT applied in acetone to the surface of adult flies (Musca domestica)

NO. FLIES	VOL. APPLIED	DDT	PER CENT MORTALITY AT HOURS INDICATED	
			24	48
	cmm.	*mgm./kgm.**		
31	0	0	3	3
25	1.0	0	4	4
45	0.2	2	17	33
40	0.4	4	15	30
20	0.6	6	15	30
70	0.8	8	46	61
80	1.0	11	60	64
23	1.2	13	56	61
30	0.8	17	60	64
26	1.0	21	50	57
25	0.8	34	80	88
25	0.4	42	76	84
5	0.8	84	100	
10	1.0	105	100	

* Average body weight of newly captured adult flies: 9.5 mgm. (70 flies).
Approximate 24 hour LD-50, 8–21 mgm. per kgm.

when injected intraabdominally into the cockroach as when given intravenously to the mammal. However, for the cockroach, in contradistinction to the mammal, DDT is found to be about as toxic when applied to the body surface as when injected. This equivalence between surface absorption and injection toxicity is not known for any toxic agent administered to mammals. The findings strongly support the assumption, therefore, that the effectiveness of DDT in insects is a function of its ready absorbability rather than of any great increase in absolute toxicity. Actually, the absolute toxicity is not unusually great when compared to that of many other agents for mammals (11).

The mechanism of this very efficient absorption and centripetal transport in the insect has not yet been carefully studied. Preliminary studies, however, indicate no impairment of absorption or centripetal transport via a leg (cockroach) which has been denervated. Nor does removal of an entire abdominal ring of chitinous exoskeleton with subsequent application of DDT caudal to the excised region prevent development of symptoms in the legs cephalad to the excised ring.

TABLE 8

Toxicity of DDT applied in acetone to the surface of adult flies (Calliphora spp.)

NO. FLIES	VOL. APPLIED	DDT	PER CENT MORTALITY AT HOURS INDICATED	
			24	48
	cmm.	*mgm./kgm.**		
26	1.0	0	4	20
30	1.0	5	13	40
29	0.2	9	23	54
36	0.4	19	45	67
45	0.6	28	40	66
20	0.8	37	85	95
20	1.0	46	85	95

* Average body weight of newly captured adult flies (large): 21.6 mgm. per fly (88 flies). Approximate 24 hour LD-50, 9–28 mgm. per kgm.

CONCLUSIONS

1. The approximate LD-50 for DDT in the cockroach (*Periplaneta americana*) is 5–8 mgm. per kgm. when injected in acetone, 10 mgm. per kgm. when applied to the body surface in acetone, 18 mgm. per kgm. when injected as emulsion and 82 mgm. per kgm. when injected in peanut oil.
2. The approximate LD-50 for DDT when applied to the body surface in acetone is 2 mgm. per kgm. for the newly emerged fly (*Musca domestica*). For the captured, older adult the LD-50 is about 8–21 mgm. per kgm. for *Musca domestica* and 9–28 mgm. per kgm. for *Calliphora*.
3. The data of various investigators, using different methods, indicate that the LD-50 values of DDT (with the exception of the newly emerged fly) for the fly, the mosquito, the cockroach, and a number of mammals (rat, rabbit, cat, dog and monkey) differ by a factor of only 3 to 5 when absorbed material is considered.
4. Death, at doses of comparable toxicity, occurs more rapidly in the fly than in the cockroach.

5. The fact that the LD-50 by surface application is about the same as that by injection, in the roach, in sharp contrast to the relationship in mammals, implies extremely efficient absorption and distribution and suggests the basic reason for the excellence of DDT as an insecticide.

6. Absorbability is the factor which differentiates the apparent toxicity of DDT for insects from that for mammals.

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THE RATE OF DISAPPEARANCE OF A MARIHUANA-ACTIVE SUBSTANCE FROM THE CIRCULATING BLOOD[1]

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Slow onset and long persistence are characteristic features of all effects of marihuana-active substances. They are observed with both crude drug extracts and pure compounds, and even after intravenous administration. The question of how the various phases of the delayed action are correlated with the concentration of the active substances in the circulation makes a study of their blood levels particularly inviting. A sufficient supply of racemic 1-hydroxy-3-*n*-hexyl-6,6,9-trimethyl-7,8,9,10-tetrahydro-6-dibenzopyran (parahexyl), a synthetic homolog of the natural hemp principles (1), was available for an attempt to estimate the blood levels of this substance in dogs at varying intervals after intravenous injection.

As there are no characteristic chemical reactions for these dibenzopyran derivatives from which to develop a chemical assay method, it was necessary to employ the ataxia test in dogs for identification of the drug in blood extracts. Relatively large amounts of blood from animals receiving high doses of marihuana were required. Lethal and near-lethal doses of parahexyl were injected as an 80 per cent solution in propylene glycol. 40 to 110 cc. of blood were taken from the jugular vein in from two to eleven hours after giving 41 to 264 mgm./kgm. of the drug and in one instance from the thoracic blood pool at autopsy eleven hours after administration of 326 mgm./kgm. In one experiment, the content of the lungs was assayed. All animals exhibited ataxia of grades IV to VI when the blood specimen was taken.

Whole blood or, in some cases, serum and clot were repeatedly extracted with ethanol, the extract was evaporated and again extracted with ethanol, and this extract after evaporation dissolved in propylene glycol. The solutions were assayed for ataxia action in dogs whose reaction had been previously standardized with known doses of tetrahydrocannabinol (2, 3). As the extracts were usually sufficient for only two or three tests, the results do not have a high degree of accuracy, but they gave a fair estimate of the order of magnitude of the parahexyl content of the blood.

As can be seen from table 1, parahexyl was always demonstrable in the blood. The amounts found varied widely between one and ten per cent of the dose administered, i.e., between less than 0.35 and 0.86 mgm. per 100 cc. The amount of drug recovered shows no consistent relationship with either the dose or the time interval between dose and sampling. The blood of the two survivors contained a much higher percentage of the administered dose than that of any of the three

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[1] This study was supported in part by a grant from Abbott Laboratories, Inc., North Chicago.

animals which succumbed. Whether or not survival was due to a slower transfer of the drug from the blood stream to the site of the lethal action is not known.

The data obtained appear to conform with what might be expected from the physico-chemical properties of these oils. It is probable that their penetration from the site of administration into tissue and cells is greatly slowed by their poor solubility in aqueous solvents. It is not usual that one or several per cent of an intravenously administered drug can be demonstrated in the blood stream several hours later.

The data available allow of no decision on the form in which the drug exists in the blood or on its distribution in the body. At least a very considerable part of the total recovered was found in the serum. This part must have been finely dispersed, since the material for extraction was taken only from the middle layer of the centrifuged serum sample.

TABLE 1

Parahexyl in blood after intravenous injection

DOG NO.	WEIGHT	DOSE	BLOOD SAMPLE		PARAHEXYL CONTENT OF BLOOD IN % OF DOSE*	GRADE OF ATAXIA	TIME TO DEATH	WEIGHT OF LUNGS, % OF NORMAL*
			Hours after injection	Cc.				
	kgm.	*mgm./kgm.*					*hrs.*	
1	9.6	41	2	90	>7	VI−	Survived	
2	9.6	146	3	85	2.8	V−	20	277
			6	40	<2.3	V		
			20		0.9†			
3	9.1	250	3½	70	10.5	IV+	Survived	
4	9.1	264	3	110	1.6	V−	18	455
5	9.1	326	44‡	44	2.4	IV	11	235

* Calculation based on an estimated blood volume equal to 7%, and lungs weight equal to 0.88% of body weight (4).

† Parahexyl content of lungs in % of dose.

‡ Blood sample taken after death.

In the one dog in which the lungs were extracted almost one per cent of the dose administered was recovered from this organ twenty hours after the drug was given. This is about one-third of what was found earlier in the total circulating blood of the same animal, but since the edematous lungs had a weight equal to about one-third of the total blood, the concentration in both was about the same. This would be consistent with the assumption that the concentration was equal in blood and edema fluid, as well as with that of an intravascular deposition of larger droplets of the drug in the lung. The former assumption would imply that the drug was capable of leaving the blood by filtration or transsudation.

The slow onset of the effect of the marihuana drugs after intravenous injection is not explained by their long persistence in the blood here demonstrated for parahexyl. Low rate of solution, intermediate storage in other organs, a slow chemical conversion into a highly dispersed form, or slow penetration to the site of action may account for the slow onset.

SUMMARY

The persistence in the blood stream of parahexyl, a synthetic marihuana-active substance, was studied in dogs. Large doses were injected by vein, and the blood concentration was determined at varying intervals by assay of the ataxia action of blood extracts in test dogs. One to ten per cent of the injected dose was found circulating between two and eleven hours after the injection. In one animal about one per cent of an intravenous dose was recovered in the lungs after twenty hours.

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FURTHER STUDIES ON THE DEPRESSANT ACTIONS OF BARBITURATES ON THE TERRAPIN CARDIAC VAGUS NERVE

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In as much as a comprehensive review of the literature on the action of barbiturates on the cardiac vagus nerve was given in a previous article by one of us (1) only one paper pertinent to the subject which has since been published will be discussed here. Gruber, Haury and Gruber (2) showed that numerous barbiturates studied by them acted qualitatively in the same manner on the cardiac vagus nerve of the turtle but their effects differed quantitatively. They were able to paralyze the vagi of all their experimental animals to which M/500 solutions of ortal sodium and evipal sodium were administered but they were able to show paralysis in only half of the experiments with the sodium salts of amytal, pentobarbital and neonal. In the other half of the experiments with the latter three drugs depression but no paralysis of the nerve was observed. Other barbiturates studied, pernoston, phanadorn, phenobarbital, nostal, and alurate, caused only depression when M/500 solutions were used. Dial, ipral and barbital had no noticeable effect on the vagus nerve in 1/500 molar solutions.

This investigation was undertaken to determine what cardiac vagal depressant effects, if any, are caused by higher dilutions of the more toxic barbiturates which had previously been studied as well as by other barbituric acid derivatives not tested in the earlier experiments.

METHOD. These experiments were performed upon terrapin of the species Chrysemys marginata. The preparation of the animal for experimentation and the apparatus used in this investigation were the same as those previously described by one of us (2).

The sodium salts of the barbiturates, dissolved in Ringer's solution, were placed in the pocket formed by the pericardial sac, thus exposing the whole heart to the drug. The pH (7.6 to 7.8) of the solution of barbiturate applied to the heart was the same as that of the control Ringer's solution. The strengths of the solutions of barbiturates employed were; 1/2000, 1/1000, 1/500, and 1/250 molar.

The vagus nerve was stimulated electrically before the application of the drug while the heart was still immersed in Ringer's solution. The control solution was then removed and replaced by the freshly made Ringer-barbiturate solution and the vagus nerve again stimulated electrically at two minute intervals. After the heart was exposed to the drug from 2 to 12 minutes, the time being dependent upon the barbiturate used and the degree of depression produced upon the vagus nerve, the Ringer-barbiturate solution was removed and the heart washed with Ringer's solution two or three times and then left immersed in a a bath of the same solution. After this the vagus nerve was again excited electrically at two minute intervals until it showed complete recovery.

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[1] This research was made possible through a grant by the McNeil Laboratories for research in science.

Results. Our experiments show conclusively that the barbiturates differ quantitatively but that qualitatively they act alike on the cardiac vagus nerve in the terrapin. In the thirty-three animals employed, 174 experiments were performed using the seven barbiturates listed in table 1.

It will be seen from the table that 1/2000 molar solution of ortal sodium caused complete block of the vagus nerve in 5 of the 6 experiments performed and M/1000 solution of ortal caused complete block of the nerve in all of the animals. The latter results are similar to those previously reported by one of us (2). Evipal produces usually only depression of the vagus with M/2000 solution but with stronger solutions (M/1000) it, like ortal, causes complete block of the nerve.

TABLE 1

In this table the barbituric acid derivatives beginning with the most efficacious are arranged in order of the intensity and rapidity of their action in depressing the cardiac vagus nerve in the terrapin — Cessation of heart beat upon vagus stimulation as in the control −+ Vagus nerve depressed less than 50 per cent as indicated by the number of heart beats during excitation +− Vagus nerve depressed over 50 per cent + Complete block of the cardiac vagus nerve as indicated by no change in the ventricular rate during excitation of the nerve

BARBITURIC ACID	NUMBER OF EXPERIMENTS	SOLUTION															
		M/2000				M/1000				M/500				M/250			
		−	−+	+−	+	−	−+	+−	+	−	−+	+−	+	−	−+	+−	+
N-Hexylethyl (Ortal)	12			1	5				6								
N - Methyl - Cyclohexenyl-Methyl (Evipal)	24	1	3	6				1	9				4				
Propyl-methyl carbinyl Allyl (Seconal)	32		1	5	1		1	6	5			1	6			1	5
Ethyl (1-Methyl butyl) (Pentobarbital)	19	1	5	2			2	3	3			1	2				
N-Butyl Ethyl (Neonal)	10							2			1	2	1				4
Sec butyl ethyl (Butisol)	39					6	5			6	7	1	1	3	8	2	
5-Ethyl-5-(1 methyl 1-butenyl (Vinbarbital, Delvinal)	38					7	3			5	10			4	7	1	1

Seconal and pentobarbital appear to be about equal in their actions in that M/2000 and M/1000 solutions usually cause depression of the nerve with the more concentrated solution sometimes producing a complete nerve block

According to our results, butisol sodium and vinbarbital (delvinal) sodium are relatively weak cardiac vagus depressant drugs. Even with as high concentration as M/250 only mild depressant effects were observed. Neonal (N-butyl ethyl) appears to be somewhat more depressant on the vagus than butisol (Sec.-butyl ethyl).

Figures 1 and 2 are presented as being typical of the results in which the vagus nerve was not completely paralyzed when the heart was treated with a solution containing a barbiturate. In figure 1 butisol sodium M/250 solution was used for the time indicated between the arrows. The vagus nerve is seen to be

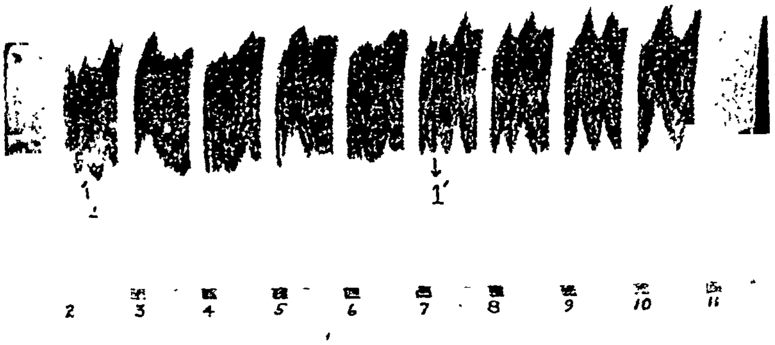

FIG. 1. Top record is that of the contractions of the ventricle of the terrapin Chrysemys marginata. The up stroke is systole and the down stroke diastole. The bottom record is the time in intervals of 30 seconds and above it a record of the duration of stimulation of the right vagus nerve.

1. Ringer's solution on the ventricle was replaced by a solution of Ringer-butisol sodium M/250.

1′. Barbiturate solution was replaced by Ringer's solution.

2 to 11 inclusive Times and duration's of stimulation of the vagus nerve. During vagal stimulation the ventricle contracted once in 5, and three times in 6 and 7. The control ventricular rate was 34 contractions per minute.

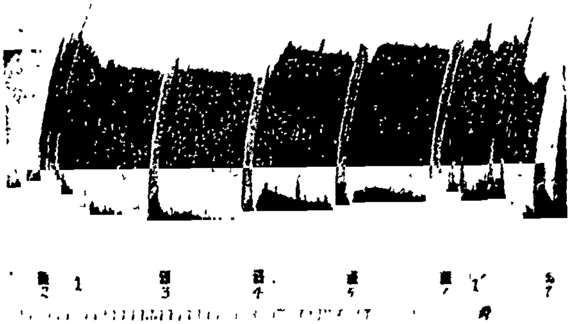

FIG 2. Top record that of the ventricle, bottom record the time in intervals of 20 seconds and above it the point and duration of stimulation of the right vagus nerve.

1. Seconal sodium solution M/1000 was substituted for the Ringer's solution bathing the heart.

1′ Barbiturate solution was replaced by Ringers' solution.

2 to 7 inclusive. Periods and durations of stimulation of the vagus nerve. While the vagus was being excited in 3 and 4 the ventricle contracted four times and in 5 and 6 it contracted 7 times during each stimulation of the vagus.

slightly depressed since three contractions of the heart occurred during vagus nerve excitation. In other experiments longer exposure of the heart to the drug appeared to have no further action.

In figure 2, M/1000 solution of seconal sodium was used between the arrows.

Complete paralysis of the nerve did not result from this dilution. Although in the control experiment, vagus stimulation had caused complete cessation of cardiac activity during the entire period of stimulation, following an 11 minute treatment of the heart by the drug vagus stimulation only slowed the heart rate from 13 to 7 beats per 20 seconds. Higher concentrations of the drug completely paralyzed the vagus nerve.

SUMMARY AND CONCLUSIONS

1. The barbiturates studied in this research act alike qualitatively but differ quantitatively in their effects on the cardiac vagus nerve in the terrapin.
2. Ortal sodium, evipal sodium, pentobarbital sodium and seconal sodium are the most toxic for the vagus nerve. Butisol sodium and vinbarbital (delvinal) sodium appear to be extremely weak cardiac vagus nerve depressants.
3. These depressant effects on the vagus nerve are temporary. After removal of the drug the vagus becomes normal in from 5 to 30 minutes depending upon the type of barbiturate used, the concentration of the drug, and the length of time that the heart has been exposed to it. The depressant action is of long duration for ortal sodium but is short for butisol sodium.

We wish to thank the following for supplying the barbiturates used in this investigation: McNeil Laboratories, Butisol sodium, and Pentobarbital sodium; Eli Lilly and Company, Seconal sodium; Parke Davis and Company, Ortal sodium; Winthrop Chemical Company, Evipal sodium; and Sharp and Dohme, Delvinal sodium.

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BEHAVIOR OF SYNTHETIC ESTERS OF STROPHANTHIDIN, THE ACETATE, PROPIONATE, BUTYRATE, AND BENZOATE, IN MAN

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It is well known that the glycosides of the strophanthus group are poorly absorbed from the gastro-intestinal tract and are only available for therapeutic use by parenteral administration. The fact that the secondary hydroxyl group of carbon atom 3 of the aglycones is very reactive chemically has been utilized in the partial synthesis of compounds of the strophanthus group. Neumann (1) prepared several esters of k-strophanthidin and tested their potency in animals. Chen and Elderfield (2) described the preparation and the intravenous potency of the acetate of k-strophanthidin, and in a subsequent report, Steldt, Anderson, Maze, and Chen (3) prepared the strophanthidin-3-*n*-propionate, -3-*n*-butyrate, and -3-benzoate, and compared their potencies by intravenous injection in cats. These were found to exhibit typical digitalis actions and their potencies differed as follows: strophanthidin, 0.33; the acetate, 0.19; the propionate, 0.26; the butyrate, 0.43; and the benzoate, 2.7 mgm. per kgm.

The present study was undertaken to explore the behavior of these esters[1] in the human subject, their potency, the speed of development of their action after intravenous injection, their absorption from the gastro-intestinal tract, the speed of their elimination, and their toxic effects. The results form the subject of the present report.

METHOD. The experiments were performed in patients with auricular fibrillation and varying degrees of heart failure. They were selected from a group of 1500 active patients in attendance at our cardiac clinics on the basis of the fact that when they were without digitalis, they tended to develop a rapid ventricular rate and were subject to a degree of failure well within the limits of safety. The method was substantially similar to that used in previous studies (4). The subject who has not been receiving digitalis for several weeks was put to bed in the hospital. The ventricular rate was counted at the apex for one minute, several times daily, under conditions of quiet and rest. This was done during a preliminary control period of at least a week in order to establish the level of the resting rate without drug. A dose of the preparation was then administered and similar counts were made at intervals of several minutes until the maximum effect was obtained, and at less frequent intervals until the effect disappeared. When the rate returned to the previous level another experiment was carried out in the same manner. It was the plan to perform as many experiments with as many preparations as possible in one and the same patient. Simultaneous observations were recorded on the general behavior of the patient, signs of toxicity, and changes in the symptoms and signs of heart failure.

Table 1 summarizes the characteristics of the six patients employed in this study.

Stock solutions of 1:1000 of the acetate, propionate, and butyrate were prepared in approximately 50 per cent alcohol and of the benzoate, 1:500 in approximately 80 per cent alcohol. These were further diluted with physiological salt solution immediately before injection in the case of the intravenous doses. The oral doses were administered on an empty stomach, well diluted in water and followed by orange juice. In one instance the benzoate was administered in the form of the crystals in a hard capsule.

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[1] We are indebted to Dr. K. K. Chen for the compounds used in this study.

Results. The effects of the drugs in all the patients are summarized in table 2. There were in all 20 doses, 13 administered orally and 7 by intravenous injection. The effects of the 4 compounds were compared in terms of their cat unit doses. The validity of this procedure is demonstrated by the analysis of the data shown in table 3. Although it is well known that different patients may show a different degree of ventricular slowing with similar doses of a drug, a comparison of intravenous doses which produced substantially similar effects shows a fairly close correspondence of the cat unit doses but wide divergence of the doses in terms of the weight of the different compounds.

TABLE 1

NAME	AGE	SEX	WEIGHT	DIAGNOSIS*	DEGREE OF HEART FAILURE
			lbs.		
Ma Do...	52	M	136	A.S., E.H., cor. scler., A.F.	Moderate
Fr Le .	68	M	118	A.S., hyper., E.H., A.F.	Slight
Be Pa. .	40	F	134	R.F., E.H., M.I., M.S., A.F.	Advanced
Al Pa.	22	M	160	R.F., E.H., M.I., M.S., A.F.	Slight
Is Po	76	M	119	A.S., E.H., cor. scler., A.F.	Moderate
Be So	51	F	108	R.F., E H., M.I., M.S., A.F.	Slight

* According to "Nomenclature and Criteria for Diagnosis of Diseases of the Heart" of the New York Heart Association (1942). A.S. (Arteriosclerosis); R.F. (Rheumatic Fever); Hyper. (Hypertension); E.H. (Enlarged Heart); Cor. Scler. (Coronary Sclerosis); M.I. (Mitral Insufficiency); M.S. (Mitral Stenosis); A.F. (Auricular Fibrillation).

The details of the response in 13 experiments were charted and are reproduced in figures 1 to 6.

Acetate. The effect of the acetate is shown in figure 1. It developed its action very rapidly after intravenous injection; the full effect within less than 30 minutes, the heart rate declining from 114 to 61 a minute. The duration of action was quite brief, the effect having practically disappeared within about 4 hours. Absorption from the gastro-intestinal tract was negligible, less than 8 per cent of the dose administered. There is the possibility that more might have been absorbed but that the very rapid elimination kept pace with a relatively slow absorption. The degree of slowing produced by the intravenous dose of 6 cat units was substantially similar to what is generally obtained by a similar dose of the digitalis glycosides.

Propionate. The effect of the propionate is shown in figure 2. Its behavior was not materially different from the acetate. A somewhat larger intravenous dose produced a peak effect in about 10 minutes. Absorption was also neglibible, a dose of 90 cat units by mouth producing about as much effect as 6 units by intravenous injection. Elimination was similarly very rapid. The very marked effect of the 9 unit dose had worn off within about an hour. This experiment suggests that the persistence of action of the propionate may be shorter than that of the acetate, although the difference may be due to other factors.

TABLE 2

DRUG	PATIENT	DOSE		ROUTE	SLOWING OF RATE	APPROXIMATE DURATION OF EFFECT	REMARKS
		C.U.*	Mgm.				
						hrs.	
Acetate	Fr Le	3	0.56	vein	18 (114–96)†	1.5	
		6	1.12	vein	53 (114–61)	4	
		37	7.0	mouth	0		
	Be Pa	6	1.12	mouth	0		
Propionate	Ma Do	6	1.54	vein	22 (113–91)	1	
		9	2.32	vein	49 (113–64)	1	
		90	23.16	mouth	20 (113–93)	4	
Butyrate	Fr Le	3	1.28	vein	24 (114–90)	2	
		30	12.8	mouth	36 (114–78)	4	Nausea, weakness, sweating, vomiting in 38 minutes; recovered 37 minutes later
Benzoate	Al Pa	4	10.8	vein	19 (118–99)	5	
		6	16.3	vein	56 (118–62)	8	Vomited in 26 minutes and in 1.5 hrs; nausea gone in 2 hrs.
		6	16.3	mouth	30 (118–88)	24	
	Be So	6	16.3	mouth	21 (95–74)	18	Nausea in 1 hour
	Is Po	6	16.3	mouth	19 (97–78)	10	
	Be Pa	6	16.3	mouth	32 (134–102)	12	Vomited in 10 mins.
		6	16.3	mouth	0		Vomited in 9 mins.
		6	16.3	mouth	52 (134–82)	48	
		6	16.3	mouth	4 (82–78)		Given 9.5 hrs. after previous dose; vomited in 1.5 hrs.
		3	8.1	mouth	19 (134–115)	10	Vomited in 40 mins.
		6	16.3	mouth	0		Nausea and vomiting in 3.5 hours; drug given in capsule

* Cat unit values taken from study by Steldt et al. (see ref. 3).

† Shows level of control rate and during full effect of drug.

Butyrate. The effect of the butyrate is shown in figure 3. The fact that this subject was the same as the one used in the case of the acetate affords a highly

TABLE 3

DOSE		DRUG	EFFECT (BEATS)	RATIO OF DOSES OF DRUGS IN TERMS OF		PATIENT
Cat unit	Mgm.			Cat units	Mgm.	
3 3	0.56 1.28	acetate butyrate	18 24	1:1	1:2.3	Fr Le Fr Le
6 6	1.12 16.3	acetate benzoate	53 56	1:1	1:14.6	Fr Le Al Pa
6 4	1.54 10.8	propionate benzoate	22 19	1:0.66	1:7.0	Ma Do Al Pa
9 6	2.32 16.3	propionate benzoate	49 56	1:0.66	1:7.0	Ma Do Al Pa

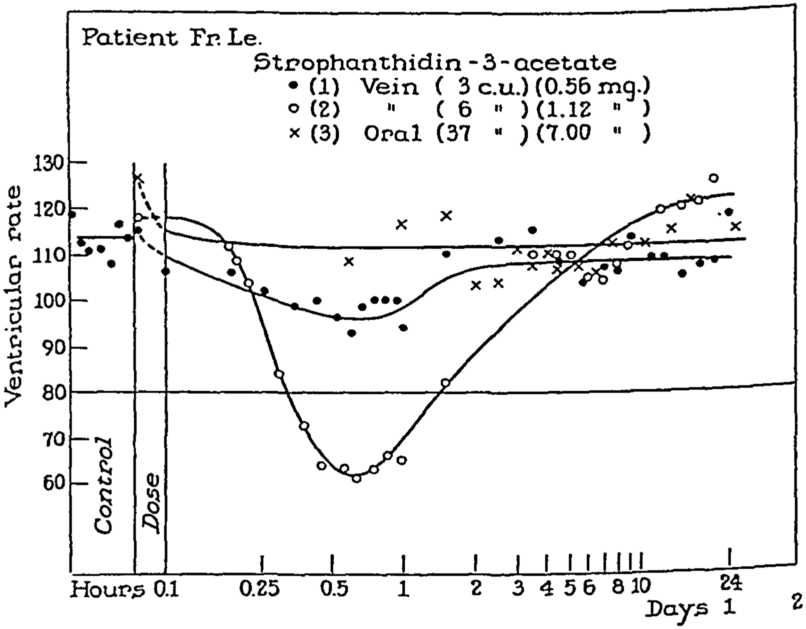

FIG. 1 (applies to all figures). Points indicate either a single ventricular count or an average of several. Only the last 7 days of the control period are represented in a space of arbitrary size. The points of the control period represent averages for each day. The rate line of the control period is drawn at a level representing the average for the 7 points. Just before the dose was injected a ventricular count was taken and recorded to the right of the first vertical line. The rate changes in the first 6 minutes are represented by broken lines since it is uncertain as to how much of these changes result from disturbances incident to the injection. After the first day points usually represent averages for the day. The numbers in parenthesis to the left of the term "vein" or "oral," refer to the order of the experiment and the number of experiments with digitalis materials which have been made in the particular patient. Time is charted logarithmically.

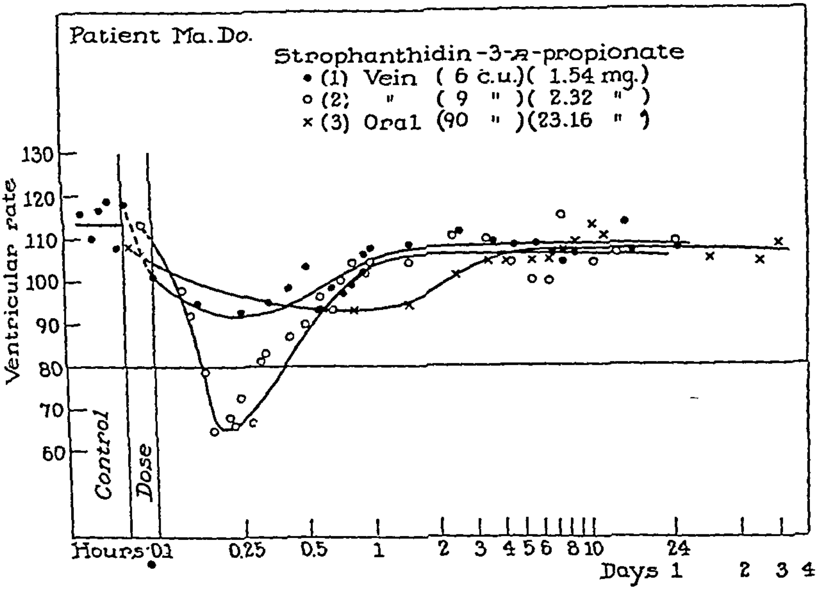
Patient Ma. Do.
Strophanthidin-3-n-propionate
• (1) Vein (6 c.u.)(1.54 mg.)
○ (2) " (9 ")(2.32 ")
× (3) Oral (90 ")(23.16 ")
Ventricular rate
130
120
110
100
90
80
70
60
Control
Dose
Hours 0.1
0.25
0.5
1
2
3
4
5
6
8
10
24
Days 1
2
3
4

FIG. 2

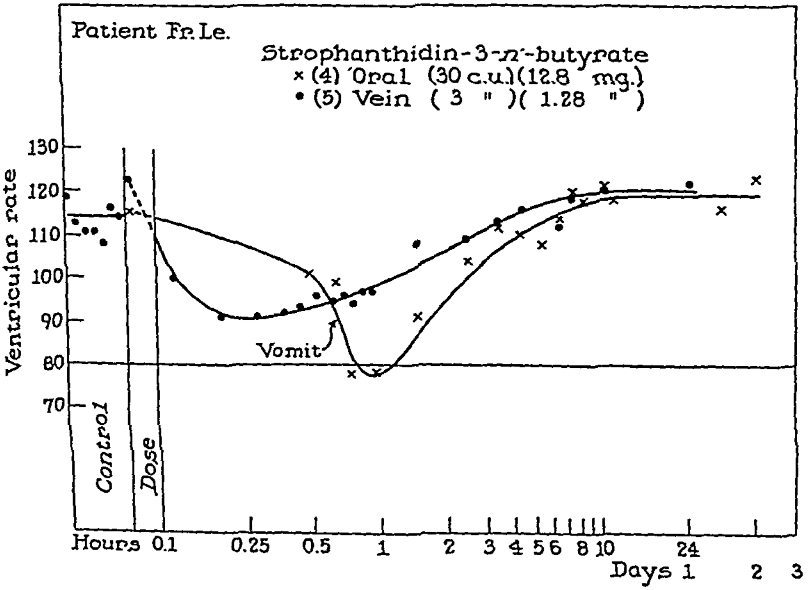
Patient Fr. Le.
Strophanthidin-3-n-butyrate
× (4) Oral (30 c.u.)(12.8 mg.)
• (5) Vein (3 ")(1.28 ")
Ventricular rate
130
120
110
100
90
80
70
Vomit
Control
Dose
Hours 0.1
0.25
0.5
1
2
3
4
5
6
8
10
24
Days 1
2
3

FIG. 3

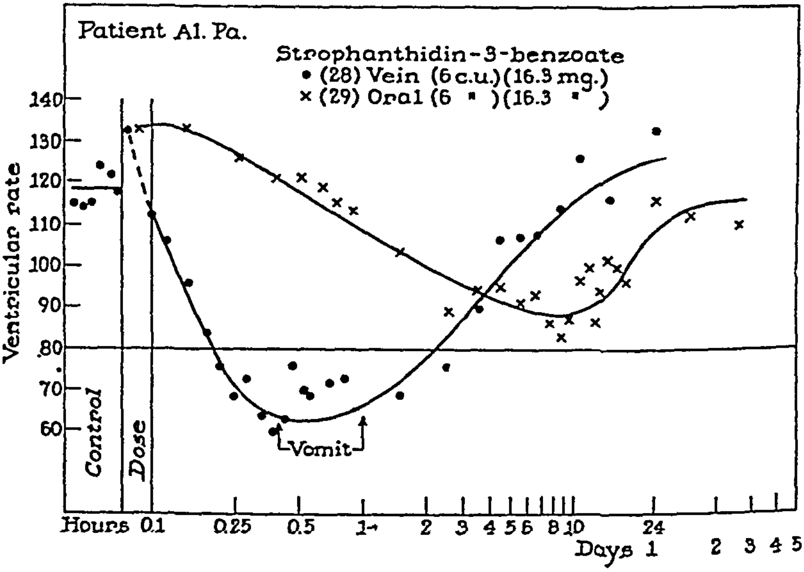

FIG. 4

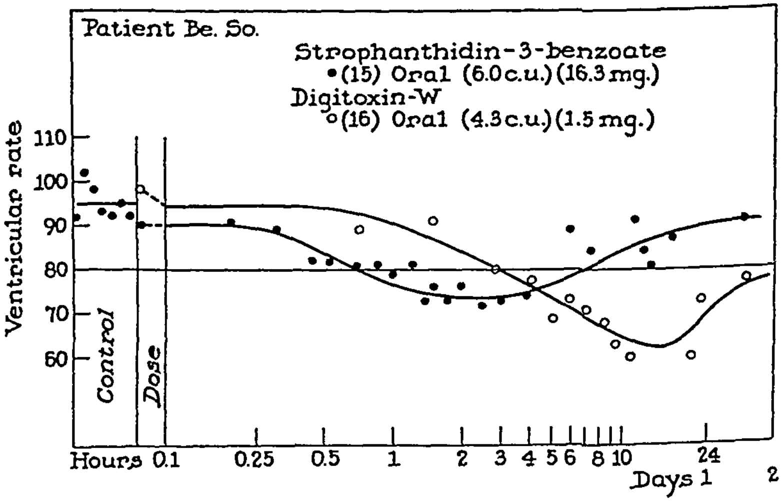

FIG. 5

favorable basis for comparison of the two compounds. The effect of the intravenous injection was very rapid and developed fully within about 15 minutes. The effect of the butyrate also wore off rapidly, within about 2 to 4 hours. This compound, however, showed a rapid and fairly high degree of absorption, the full effects of an oral dose developing within about an hour. The oral dose of 30 cat units (12.8 mgm.) produced vomiting in 38 minutes with recovery about half an hour later. In view of the rapid absorption it is not possible to know whether the emetic action was local or systemic. Doses of digitalis glycosides as high as 12.8 mgm. frequently cause vomiting in man by local action (5).

Benzoate. Figure 4 shows that by intravenous injection, the benzoate developed its full effects in a manner similar to that of the previous compounds. Its duration of action was longer, about 8 hours. It showed an unusual degree of absorption, nearly the full effect developing within about 2 to 3 hours. It is noteworthy that a high degree of digitalization resulted from an oral dose of only 6 cat units. The dose by intravenous injection was more effective than by oral administration; the intravenous dose lowered the ventricular rate to 62 as against 88 after the oral dose. The intravenous dose also caused vomiting at the peak of its effect. These findings suggest that the absorption was not complete, although, again, the absorption may have been complete, the difference being accounted for by the fairly rapid elimination.

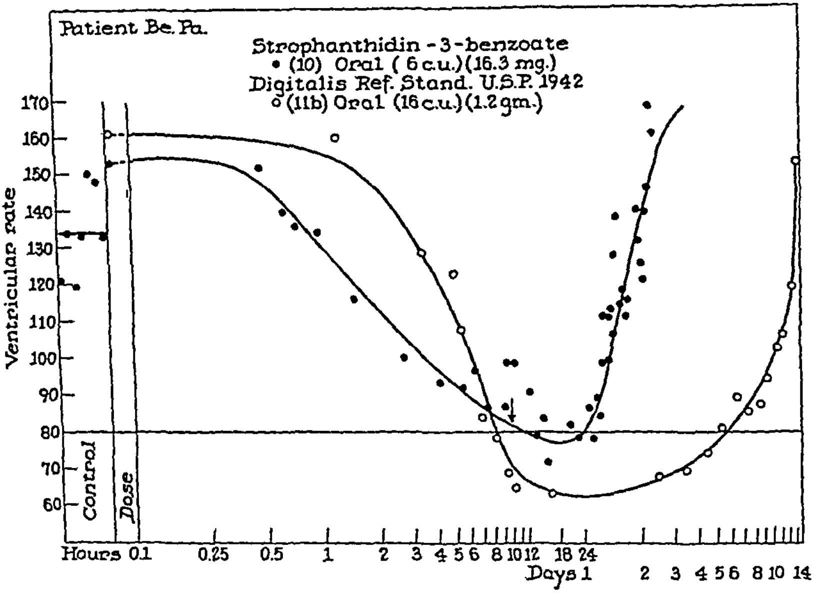

FIG. 6

The high degree of absorption of the benzoate is shown in another patient (fig. 5) in whom the peak effect of an oral dose of digitoxin was nearly matched by a 40 per cent larger dose of the benzoate.

The oral dose of the benzoate was compared with digitalis leaf in a third patient (fig. 6). Its absorption was clearly better than that of digitalis. A dose of 6 cat units of the benzoate produced nearly the same peak effect as 16 units of digitalis. In the first few hours, when the effect of the digitalis was negligible, nearly the full effect of the dose of the benzoate had already developed. The oral dose of the benzoate frequently caused vomiting, evidently due to a local action in some instances since it occurred within about 10 minutes. It migh be mentioned that in this patient, who was in advanced failure, the gastro-intestinal tract was unusually sensitive, since she frequently vomited other medications, as well as shortly after meals. The slowing of the ventricular rate was attended by fairly pronounced subjective improvement in the symptoms of heart failure, but attempts to maintain the improvement by repetition of the doses (arrow on fig. 6) proved unsuccessful because of her tendency to lose the drug through vomiting.

SUMMARY

1. The behavior of 4 synthetic esters of k-strophanthidin, the acetate, propionate, butyrate, and benzoate, was explored in a group of patients with auricular fibrillation and heart failure.

2. A study was made of their potency, speed of action by intravenous injection, persistence of action, absorption from the gastro-intestinal tract, and toxic effects.

3. The relative potencies of the esters by intravenous injection in cats and by intravenous injection in man are similar.

4. The esters produce essentially the same kind of effects as the digitalis glycosides in man. In doses corresponding to those of the digitalis glycosides, they slow the ventricular rate in auricular fibrillation, and produce nausea and vomiting by both a systemic action and a local action in the gastro-intestinal tract. There is indication that in suitable doses they may exert a similar action on the symptoms of heart failure.

5. The esters develop their full action after intravenous injection in man, more rapidly than the digitalis glycosides in common use, namely, within 10 to 30 minutes.

6. The persistence of action of the esters is brief, the effects wearing off within a few hours. There is indication, although this point is in need of further study, that the benzoate persists somewhat longer than the others.

7. The absorption of the acetate and propionate from the gastro-intestinal tract of man appears to be negligible. The butyrate shows fairly rapid absorption, producing its full effects within about an hour, although the ratio of the oral to intravenous dose for a similar effect resembles that of digitalis. The benzoate is unusually well absorbed, and there is indication that its absorption may be nearly complete, approximating the behavior of digitoxin in this respect.

8. Although the glycosides of the strophanthus group are so poorly absorbed from the gastro-intestinal tract as to preclude their use by oral administration, some of the esters of their aglycones are better absorbed than most of the glycosides of the digitalis series. Their speed of action offers interesting possibilities for application in cardiac therapy. The esters deserve more extensive investigation.

These studies were supported in part by the Digitalis Fund of Cornell University Medical College, which includes contributions from Eli Lilly and Company, Wyeth, Inc., Lederle Laboratories, Inc., E. R. Squibb & Sons, Varick Pharmacal Co., The Wm. S. Merrell Company, and the David, Josephine, and Winfield Baird Foundation.

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THE CHEMOTHERAPEUTIC PROPERTIES OF 5-NITRO-2-FURALDEHYDE SEMICARBAZONE (FURACIN)

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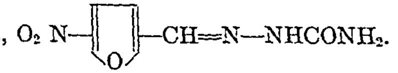
A previous report (1) has shown that a nitro group in the 5-position in the furan ring confers definite bacteriostatic and bactericidal activity on a variety of 2-substituted derivatives. At the present time, sixty-one such compounds have been examined for possible therapeutic value in experimental infections in mice. One of these, 5-nitro-2-furaldehyde semicarbazone demonstrated a definitely beneficial effect in the treatment of both bacterial and trypanosome infections in mice by either oral or subcutaneous administration. To date, none of the compounds unrelated to the semicarbazone and previously reported (1) as derivatives of nitrofuran, nitrofuroic acid or nitrofurfuryl alcohol has shown therapeutic action *in vivo* although practically all of them produced definite bacteriostatic and bactericidal action on both Gram positive and Gram negative organisms *in vitro*.

The literature on earlier studies of the antibacterial action of furan compounds (1) has failed to reveal either the unique action of the nitro group in activating a variety of simple furan derivatives, *in vitro*, or the equally singular *in vivo* therapeutic effect produced by the semicarbazone group substitution in nitro furaldehyde. For these reasons, as well as the wide interest and possibilities in new therapeutic agents of known structure, the antibacterial action of 5-nitro-2-furaldehyde semicarbazone has been extensively studied *in vitro* and in several different infections in mice. While the present paper is intended chiefly as a report on these factors, certain studies on the toxicity of the compound necessary for an evaluation of its therapeutic action in mice are also given. Krantz and Evans (2) have, meanwhile, made a detailed investigation of the toxicity and pharmacological properties of the compound.

STRUCTURE AND PROPERTIES. 5-nitro-2-furaldehyde semicarbazone is represented by the structural formula,  It is a yellow, needle-like solid, slightly soluble in water (1–4200), somewhat more soluble in 95 per cent ethanol, and acetone, but virtually insoluble in ether. Analyses of various preparations of the compound show that the carbon, hydrogen and nitrogen content agree closely with calculated values. The melting point of the compound is not sharp; depending on the rate of heating and possibly other factors, the sample when tested in the usual capillary melting tube is observed to darken, becomes tarry and finally liquefies in the range of 227–241°C. The compound in aqueous solution has an absorption peak at 3750 A. In the presence of alkalies an intense red color is formed in aqueous solution, which disappears upon acidification.

Experimental. The *in vitro* studies were performed with the organisms listed in table 1 using beef infusion broth wherever good growth was obtained, and supplemented with 10 per cent normal horse serum for the pneumococcus. The same medium containing 2 per cent glucose was employed for tests on the anaerobes listed. Tests on *N. gonorrhoeae* and *N. intracellularis* were done however in brain-heart infusion broth containing 10 per cent normal horse serum.

The inoculum, in most cases, consisted of 20,000 organisms from fresh cultures in the same medium used in the test. However, the inoculum used in tests on the *Neisseria* strains and the anaerobes was 0.1 cc. of fresh cultures of these organisms.

Ten cc. quantities of the desired concentrations of the drug in the above media were inoculated as above. Incubation was carried out for four days at 37°C with daily observations of the results The tests on the anaerobes were incubated in Novy jars under the proper conditions. Estimations of bacteriostatic action were made by visual comparison of the test samples with proper controls. The twenty-four hour readings in table 1 represent the minimum concentration of the compound which limited growth to 50 per cent of the control growth in that time period. The four-day readings denote the minimum concentration which prevented any visible growth.

TABLE 1

Bacteriostatic activity of 5-nitro-2-furaldehyde semicarbazone in broth

ORGANISM	MINIMUM BACTERIOSTATIC CONCENTRATION IN 24 HOURS (50 PER CENT CONTROL)	MINIMUM CONCENTRATION PRODUCING COMPLETE INHIBITION FOR 4 DAYS
S. aureus (Smith)	1-100,000	1-80,000
S. hemolyticus (C203)	1-100,000	1-10,000
S. viridans	1-5,000	>1-5,000*
S. fecalis	1-40,000	1-5,000
S. anhemolyticus	1-40,000	1-10,000
D. pneumoniae I	1-40,000	>1-5,000*
N. gonorrhoeae	1-100,000	1-100,000
N. intracellularis	1-100,000	1-40,000
Ps. pyocyanea	>1-5,000*	>1-5,000*
E. coli	1-100,000	1-80,000
S. schottmuelleri	1-100,000	1-100,000
S. paratyphi	1-100,000	1-100,000
E. typhosa	1-200,000	1-100,000
S. dysenteriae	1-200,000	1-100,000
Proteus vulgaris	1-20,000	1-10,000
Cl. welchii	1-20,000	1-5,000
Cl. tetani	1-20,000	1-5,000
Cl. novyi	1-200,000	1-40,000
M. tuberculosis (var. hominis-607)	1-200,000 (48 hours)	1-5,000
M. tuberculosis (Gary)	1-500,000 (30 days)	1-40,000 (30 days)

* Maximum solubility in broth

The tests on *M. tuberculosis* were run in 25 cc. of 5 per cent glycerol broth containing various concentrations of the compound in 50 cc. Erhlenmeyer flasks. The inoculum was a particle of the surface growth of a similar culture floated on to the surface of the test medium. Incubation was at 37°C for four days with strain 607 and for thirty days with the Gary strain. Estimations of drug activity were made by comparing the size of the surface growth in the test broth with control growth.

The toxicity studies were done on the CFW strain of white mice. The drug was administered by stomach tube as a suspension in 10 per cent gum acacia. Observation was continued on survivors for a week. Calculation of the LD50 was done according to the formula of Reed and Muench (3).

The *in vivo* tests with bacterial infections were done on the CFW strain of mice. Six hour cultures of the various species listed in tables 3 and 4 in brain-heart infusion broth containing 10 per cent defibrinated rabbit blood were diluted in broth or 5 per cent mucin so that the quantity and dilutions indicated in the tables contained approximately 100 to 10,000 lethal doses of the organism. This was usually 0.5 cc. of a 10^{-6} or 10^{-4} dilution respectively as previously determined by titration with higher dilutions. The infection was inoculated into the peritoneal cavity. Treatment was started immediately with the drug being administered by stomach tube as a suspension in 10 per cent gum acacia, and repeated as indicated in the tables.

Trypanosoma equiperdum infections in mice and rats were also treated with the drug. In both cases, blood from an infected mouse or rat was diluted with broth so that 1.0 cc. inoculated intraperitoneally contained 150,000 organisms. Treatment of mice was instigated by stomach tube twenty-four hours following inoculation. Rats were treated on the third day of the infection.

RESULTS OF IN VITRO TESTS. Reference to table 1 will show that 5-nitro-2-furaldehyde semicarbazone is bacteriostatic for a number of Gram-positive and Gram-negative bacteria in rather low concentrations. *Ps. pyocyanea* was the only organism tested on which the drug had no effect even in a saturated solution. The growth of *S. viridans* and *D. pneumoniae* was inhibited for 24 to 48 hours but the maximum concentration possible failed to completely stop growth for four days. In general, the compound appeared to be quite effective in limiting the growth of the other Gram-positive and Gram-negative organisms tested with the above exceptions. It was approximately ten times more active on *Cl. Novyi* than on the other two anaerobic species tested. The effect on *M. tuberculosis* was more pronounced with the slower growing pathogenic strain (Gary).

Previous work (1) has shown that concentrations of nitrofuran compounds which inhibit all growth for four days were also bactericidal and this was shown to be the case also with 5-nitro-2-furaldehyde semicarbazone. It was also shown that larger inocula somewhat reduced the bacteriostatic and bactericidal action of the compound but did not abolish it. Further unpublished work revealed that the same effect was produced by whole blood and by serum. However, the antibacterial activity of the compound on the growth of *E. coli* is considerably enhanced in the presence of a synthetic medium devised by Long and Bliss (4).

TOXICITY. Table 2 shows the LD50 of seven lots of 5-nitro-2-furaldehyde semicarbazone obtained from oral administration to mice. A composite LD50 calculated by treating all the mice used as a single test is given for comparison. The last column presents the data and the calculated LD50 obtained from the administration of a mixture of the seven lots. It will be noted that a larger variation was obtained in the LD50 from one sample to another than is to be expected from a pure compound. No explanation is available at the present time as the analysis for each lot showed the values for C, H, O and N to be very close to the calculated values. However, a comparison of the LD50 value for the composite of all seven lots with the value obtained for the mixture of the same samples shows exceptionally good agreement.

The insolubility of the compound precludes the determination of an LD50 by intravenous administration without the use of solvents and this was not attempted for this report. However, 0.5 cc. to 1.0 cc. quantities were administered by this route to mice and rats and a description of the symptoms produced is given below.

The compound was injected subcutaneously into mice and rats as a 10–20 per cent suspension in gum acacia in doses up to 3 grams per kilogram of body weight. This also failed to cause a sufficient number of deaths to allow a calculation of an LD50 but did produce symptoms described in the next paragraph. The most likely explanation for this failure of such large doses to cause death is again the poor solubility of the compound which probabily permits only a very slow absorption from the site of injection.

The most prominent symptom produced by the administration of 5-nitro-2-furaldehyde semicarbazone was hyperirritability. This is observed following oral doses of 300 mgs./k. or more and was present after the intravenous injection of 1.0 cc. in rats and the larger subcutaneous doses in both mice and rats. Death following oral doses occurred from two to forty-eight hours after feeding, the majority of animals dying in two to twelve hours. Death was preceded by increased hyperirritability, tremors and convulsions and seemed to be the result of respiratory failure. Doses of 200–300 mgs./k. repeated every eight hours for four days produced a similar sequence of toxic responses.

TABLE 2

Toxicity of 5-nitro-2-furaldehyde semicarbazone from a single oral dose

	No. mice dead/No. mice used								
DOSE	LOT 1	LOT 2	LOT 3	LOT 4	LOT 5	LOT 6	LOT 7	COMPOSITE	MIXTURE
mgs./k.									
100	0/10	2/10						2/20	
200	0/10	1/10	0/10	0/10				1/40	0/12
300	0/10	3/10	0/10	4/20	1/8	0/14	4/20	12/92	0/12
400	2/10	2/10	4/20	6/20	1/8	6/20	8/20	29/108	3/12
500	11/20	10/20	9/20	7/20	1/16	9/20	4/20	41/136	3/12
600	7/10	8/10	9/20	7/14	5/16	11/20	5/20	52/110	7/12
700			9/10	6/8	6/16	6/8	7/10	34/52	6/12
LD50 mgs./k	460	468	548	521	673	531	582	545	587

HISTOPATHOLOGY. Mice and rats fed single doses of 150, 200 and 300 mgs./k. were sacrificed thirty-six to seventy-two hours after feeding and specimens of vital organs removed and studied microscopically for the presence of possible tissue damage due to the compound.

This study was essentially negative. Sections of brain, heart, lungs, bone, kidney, spleen and of the gastro-intestinal tract appeared normal. The only possibly significant finding was a slight cloudy swelling of the liver in some of the animals.

Similar studies on tissues of mice and rats fed 100–150 mgs./k. of the compound every eight hours for four to six days also showed only the effect on the liver noted above. When the compound was incorporated into the daily rations of mice so that each animal received 5 mgs. per 20 gm. of body weight per day for four to six days, the histological examination failed to show any abnormalities in the above-mentioned tissues, the liver also appearing normal.

The subcutaneous injection of 3 gms./k. of the compound, although it did not cause the death of mice and rats, did produce definite lesions in the liver and kidneys. The sections of liver showed, "an almost complete loss of the normal architecture with a swelling of all the cells and a very marked cytoplasmic degeneration. There is also considerable nuclear degeneration." The sections of the kidney revealed, "a marked degeneration of the tubular epithelium, particularly in the loops of Henle, and the central portions of the tubules contain a precipitated protein material." The other tissues of these animals were normal in appearance.

These studies as well as the more extensive work of Krantz and Evans failed to show any definite physiological or histological evidence as to the cause of the toxic symptoms produced by the oral administration of the compound. However, massive subcutaneous doses did produce a severe toxic hepatitis and an extensive degeneration of the tubular epithelium of the kidney.

RESULTS OF ORAL THERAPY OF INFECTIONS IN MICE. The experiments in the following tables which are discussed here are typical examples and have been repeated five or six times on each infection.

Staphylococcus aureus. It will be noted in table 3 that a single oral dose of 50 mgs./k. of 5-nitro-2-furaldehyde semicarbazone daily for three days protected 23.3 per cent of the mice infected with 10,000 times the LD50 of *S. aureus.* When the dose of the compound was doubled, approximately twice as many animals survived the infection. The maximum therapeutic effect 63.3 per cent was reached, however, from a single, daily, oral dose, when 150 mgs./k. was administered. Further increases in dosage up to 250 mg./k. seemed to have an adverse effect on the survival rate reducing it to about the same percentage obtained with the smaller doses.

Table 3 also demonstrates that the addition of a second dose of equal size twelve hours after the first dose failed to increase the survival rate beyond that obtained with a single dose. Again the maximum effect was noted following a dose of 150 mgs./k. Three doses at eight hour intervals produced a marked reduction in the survival rate regardless of whether the dose was large or small.

The effect of the size of the infecting inoculum on the protective action of the compound is demonstrated in table 3. Here it will be noted that a one-hundred-fold reduction in the inoculum had a very favorable action on the effectiveness of the compound, doubling the survival rate of the smaller doses and in general removing the apparent optimum dosage effect at 150 mgs./k. noted above.

Streptococcus hemolyticus. 5-nitro-2-furaldehyde semicarbazone was also effective in protecting mice infected with hemolytic streptococci. The data in table 3 show that single, daily, oral doses of from 50 to 150 mgs./k. for three days protected from 26.7 to 76.7 per cent of the animals. However, an adverse effect was noted as before when either a large or a small dose was administered at twelve or eight hour intervals. A comparison with the previous data on *S.*

TABLE 3

Oral therapy of S. aureus and S. hemolyticus infections in mice with 5-nitro-2-furaldehyde semicarbazone

Organisms: *S. aureus* (Smith) and *S. hemolyticus* (C-203).
Culture: 6 hours in 10% rabbit blood broth
Infection: injected intraperitoneally
Treatment: orally immediately after infection

NO. OF MICE	DOSE MGS./K.	S. AUREUS		NO. OF MICE	S. HEMOLYTICUS	
		Culture dilution	Per cent survival*		Culture dilution	Per cent survival*
Therapy: single daily dose for 3 days						
30	50	10^{-4}	23.3		10^{-5}	26.7
30	100	10^{-4}	40.0		10^{-5}	70.0
30	150	10^{-4}	63.3		10^{-5}	76.7
30	200	10^{-4}	36.7		10^{-5}	63.3
30	250	10^{-4}	40.0		10^{-5}	70.0
30	50	10^{-6}	53.3		10^{-7}	53.3
30	75	10^{-6}	80.0		10^{-7}	60.0
30	100	10^{-6}	83.3		10^{-7}	53.3
30	150	10^{-6}	70.0		10^{-7}	
Therapy: two doses at 12 hour intervals for 1 day						
30	25	10^{-4}	13.3		10^{-5}	10.0
30	50	10^{-4}	20.0		10^{-5}	23.3
30	75	10^{-4}	13.3		10^{-5}	30.0
30	100	10^{-4}	43.3		10^{-5}	40.0
30	150	10^{-4}	60.0		10^{-5}	50.0
Therapy: three doses at 8 hour intervals for 1 day						
30	25	10^{-4}	16.7		10^{-5}	36.7
30	50	10^{-4}	3.3		10^{-5}	40.0
30	75	10^{-4}	36.7		10^{-5}	50.0
30	100	10^{-4}	36.7		10^{-5}	36.7
Untreated Controls						
90		10^{-4}	0	90	10^{-5}	0
37		10^{-5}	0	30	10^{-6}	0
75		10^{-6}	0	60	10^{-7}	0
45		10^{-7}	42.2	25	10^{-8}	16.0
45		10^{-8}	46.7	25	10^{-9}	52.0

* 10 days after inoculation

aureus shows that the compound is more effective on the streptococcic infection, although the difference is not marked. Also, the optimum dosage effect noted at 150 mgs./k. in therapy of *S. aureus* infection, is not so apparent with this infection, for while the highest survival rate was obtained with 150 mgs./k., the increase at this dose is probably not significant. In contrast also, a decrease in the size of the inoculum of the infecting organism failed in this case to increase the effectiveness of the compound as was the case with *S. aureus* infections.

Pneumococcus. Experiments similar to those on *S. aureus* and *S. hemolyticus* were done using types I, II, and III *D. pneumoniae.* The compound had no significant effect on the course of pneumococcus infections in mice regardless of the size and number of doses of drug administered, or of the infecting inoculum.

Salmonella infections. The data in table 4 show that 5-nitro-2-furaldehyde semicarbazone is also effective in protecting mice infected with the Gram negative species *Salmonella schottmulleri* and *Salmonella aertrycke.* Both infections are about equally sensitive to the effect of the compound, comparative doses producing approximately equal survival rates. As was noted with other infections discussed previously, the maximum effect on an infection with approximately 10,000 lethal doses was obtained with a single, daily dose of 150–200 mgs./k. which protected around 60 per cent of the mice with either infection. As previously noted also, multiple doses failed to increase the survival rate or definitely decreased the protective action. In both of these infections the drug was markedly more effective when the infecting inoculum was reduced to 100 lethal doses. A further comparison of these data with the results obtained on therapy of staphylococcus and streptococcus infections reveals the compounds to be about equally effective on the Gram negative species *in vivo* as on these two pyogenic forms when the larger inoculum was employed and slightly more effective on the smaller inoculum of the Gram negative species.

Trypanosoma equiperdum. Table 5 demonstrates the effectiveness of 5-nitro-2-furaldehyde semicarbazone in the therapy of *Trypanosoma equiperdum* infections in mice and rats. In mice, a single oral dose of 40 mgs./k. of the drug given twenty-four hours after infection, when numerous trypanosomes could be found in the blood, completely cleared the blood in 24–36 hours, although only 10 per cent of the mice survived the test period. In the other animals, the infection was not cured and relapse occurred in from four to fourteen days. However, larger doses increased the survival rate and the highest dose tried, 150 mgs./k., protected 73.3 per cent of the test animals for the entire test period of three weeks. At the end of this time, a further attempt to demonstrate trypanosomes was made. Five animals were killed and their spleens removed and minced in a small quantity of broth which was then injected into the peritoneal cavity of normal mice. All of these latter animals survived for three weeks and no trypanosomes were observed in stained blood smears made at frequent intervals.

The compound was more effective in therapy of the infection in rats. A dose of 20 mgs./k. cleared the trypanosomes from the blood in 24–36 hours and the survival rate from comparative doses was greater than with mice. In addition, 100 mgs./k. protected 100 per cent of the animals so treated, the only instance in all the therapy presented here in which complete protection of all animals on a given dose was obtained.

TABLE 4

Oral therapy of S. schottmülleri and S. aertrycke infections in mice with 5-nitro-2-furaldehyde semicarbazone

Organisms: *S. schottmülleri* and *S. aertrycke.*
Cultures: 6 hours in 10% rabbit blood broth.
Infection: injected intraperitoneally.
Treatment: orally immediately after infection.

NO. OF MICE	DOSE MGS./K.	S. SCHOTTMÜLLERI		NO. OF MICE	S. AERTRYCKE	
		Culture dilution	Per cent survival*		Culture dilution	Per cent survival*
Therapy: single daily dose for 3 days						
60	50	10^{-5}	13.3		10^{-5}	28.3
60	100	10^{-5}	23.3		10^{-5}	18.3
60	150	10^{-5}	58.3		10^{-5}	60.0
60	200	10^{-5}	61.7		10^{-5}	63.3
60	250	10^{-5}	33.3		10^{-5}	40.0
30	50	10^{-7}	10.0		10^{-7}	36.7
30	75	10^{-7}	83.3		10^{-7}	56.7
30	100	10^{-7}	73.3		10^{-7}	80.0
30	150	10^{-7}	93.3		10^{-7}	93.3
Therapy: 2 doses at 12 hour intervals for 1 day						
30	25	10^{-5}	0		10^{-5}	3.3
30	50	10^{-5}	10.0		10^{-5}	20.0
30	75	10^{-5}	26.7		10^{-5}	23.3
30	100	10^{-5}	26.7		10^{-5}	0
30	150	10^{-5}	66.7		10^{-5}	6.7
Therapy. 3 doses at 8 hour intervals for 1 day						
30	25	10^{-5}	6.7		10^{-5}	20.0
30	50	10^{-5}	20.0		10^{-5}	30.0
30	75	10^{-5}	40.0		10^{-5}	43.3
30	100	10^{-5}	56.7		10^{-5}	53.3
Untreated Controls						
120		10^{-5}	0	120	10^{-5}	0
15		10^{-6}	0	20	10^{-6}	0
60		10^{-7}	0	60	10^{-7}	0
28		10^{-8}	3.6	20	10^{-8}	35.0
10		10^{-9}	20.0	10	10^{-9}	30.0

* 10 days after inoculation.

Spirochetal Infections. The compound failed to influence the course of relapsing fever infections with *Spirocheta novyi* in rats. The appearance of spirochetes in the blood following intraperitoneal inoculation could not be prevented by oral doses up to 300 mgs./k., and repeated doses neither limited the number found in the blood nor affected the length of time they remained as compared to control animals.

Preliminary experiments with *Treponema pallidum* indicate that the compound is active on this spirochete. Using the method of Eagle (5), spirocheticidal activity was observed *in vitro*, and oral therapy has affected both the darkfield findings and the appearance of scrotal lesions in rabbits. Detailed information on these experiments will be published when the experiments are completed.

TABLE 5

Oral therapy of T. equiperdum infection in mice and rats with 5-nitro-2-furaldehyde semicarbazone

Organism: *Trypanosoma equiperdum.*
Infection: 150,000 organisms injected intraperitoneally.
Treatment: mice: single oral dose 24 hours after inoculation.
rats: single oral dose on third day after inoculation.

NO. OF MICE	DOSE	SURVIVAL FOR 3 WEEKS	NO. OF RATS	DOSE	SURVIVAL FOR 3 WEEKS
	mgs./k.	*per cent*		*mgs./k.*	*per cent*
10	10	0	10	10	0
40	20	0	10	15	0
20	30	0	15	20†	6.6
40	40*	10.0	30	25	23.3
20	50	15.0	10	30	0
20	60	50.0	15	40	33.3
20	80	40.0	20	50	85.0
40	100	62.5	15	60	33.3
15	150	73.3	15	70	66.6
130	None	0	15	80	86.6
			15	90	86.6
			35	100	100.0
			40	None	0

* Cleared blood of 60% of mice.
† Cleared blood of all rats.

Diet Therapy. Mice infected with approximately 10,000 lethal doses of *Streptococcus hemolyticus* were also treated with 5-nitro-2-furaldehyde semicarbazone administered as a part of the daily rations. The compound was mixed thoroughly with ground Purina mouse ration in the percentages shown in table 6. Immediately following inoculation the mice were given access to the drug-diet mixtures for periods from two to four days as indicated. Previous experiments with normal mice had demonstrated the percentages of compound and the quantity of food to be used in order to administer the calculated dosage of compound per mouse each day as indicated in table 6. It has been noted previously that normal mice fed the drug as a part of the daily ration for four days showed no symptoms of toxicity, nor did their tissues reveal any damage which could be attributed to the compound.

The data in table 6 indicate that the compound produced a therapeutic effect by this method of administration comparable to the results obtained when similar doses are given once a day by stomach tube, although the latter method was somewhat more effective. Again it was found that a daily total of drug equal to 150–200 mgs./k. of body weight gave a higher percentage of survival than larger quantities. Further experiments with this method of administration in which the drug-diet will be instigated before inoculation will be presented at a later date.

Subcutaneous therapy. It has already been shown that 5-nitro-2-furaldehyde semicarbazone is much less toxic when injected subcutaneously or intramuscularly than by oral administration. It has been suggested that this decrease in toxicity may be due to the low solubility of the compound, the intestinal contents providing a sufficiently larger quantity of solution than would be available at the site of an injection of the material so that absorption in the former instance is more rapid.

TABLE 6

Diet therapy of S. hemolyticus infection in mice with 5-nitro-2-furaldehyde semicarbazone

Organism: *Streptococcus hemolyticus.*
Culture: 6 hours in 10% rabbit blood—brain heart infusion broth.
Infection: 1.0 cc 10^{-5} culture dilution injected intraperitoneally.
Treatment: administered as part of daily ration.

NO. OF MICE	DOSE MOUSE MGS./K./DAY	PER CENT OF DIET	NO. DAYS FED	NO. SURVIVING IN DAYS										SURVIVAL
				1	2	3	4	5	6	7	8	9	10	*per cent*
30	150	0.09	3	22	22	22	22	20	20	18	18	18	18	60.0
30	200	0.125	2	29	24	21	18	17	16	16	16	16	16	53.3
30	200	0.125	4	29	25	21	21	19	19	19	19	19	19	63.3
30	300	0.180	3	17	15	14	13	13	13	13	13	13	13	43.3

Preliminary experiments on the therapeutic effect of the compound by either the subcutaneous or intramuscular route have demonstrated that much larger quantities of the compound are required for protection of mice infected with hemolytic streptococci or *Trypanosoma equiperdum.* In the former infection, single doses of 500 mgs./k. of body weight gave 60 per cent survival, while 100 mgs./k. once daily for three days produced a survival rate of 90 per cent in trypanosomiasis. Further experiments using several solvents in an effort to increase absorption will be reported later.

DISCUSSION. The foregoing experiments illustrate the therapeutic action of 5-nitro-2-furaldehyde semicarbazone orally on the course of infections in mice with *Staphylococcus aureus, Streptococcus hemolyticus, Salmonella schottmülleri* and *Salmonella aertrycke.* Similar experiments have been performed on a total of two to three thousand mice with each infection using certain variations of the dosage and treatment intervals described here with approximately the same results.

In view of this rather extensive trial the activity of the compound seems to be definitely established. However, no chemical test for the presence of the compound in body fluids is available and attempts to demonstrate antibacterial activity in the blood of treated animals using the methods of Rammelkamp (6) and Rake and Jones (7) for the assay of penicillin have failed. This imposed a purely arbitrary selection of dosage and treatment interval so that these data possibly do not represent the maximum effectiveness obtainable with an intelligent use of the drug based on knowledge of its absorption, elimination and mode of action. For example, the consistent failure of repeated administration of the compound to increase the survival rate beyond that obtained with single doses as noted in these experiments may not be the result of the action of the compound, and adjustment of dosage and time of administration based on knowledge of blood levels and elimination may explain this apparent discrepancy.

It is difficult, though, to explain the apparent maximum effect of 60–70 per cent survival in certain infections obtained with a single, daily dose of 150–200 mgs./k. of body weight while larger doses either failed to increase the percentage survival or even reduced it. This is rather unexpected since doses of up to 300 mgs./k. do not produce a high mortality rate in normal mice nor do they cause toxic symptoms other than hyperirritability. In addition, infected mice treated with these larger doses which fail to survive, die with symptoms of infection, rather than toxicity, and blood cultures revealed the presence of numerous organisms similar to the findings with untreated animals.

The relatively poor bacteriostatic effect of the compound on *D. pneumoniae* and the absence of any bactericidal effect *in vitro* suggest that the negative results obtained in the attempted therapy of pneumococcus infections in mice are probably due to the lack of susceptibility of this organism to the drug and further information leading to more intelligent dosage will probably not increase the survival rate from pneumococcus infections.

The adverse effect of a large inoculum of the infecting organism on the antibacterial activity *in vivo* has been noted. This was more outstanding in the infections with the Gram negative organisms. The same action of large inocula of organisms has been previously noted on the *in vitro* action of the compound and further work has shown a similar effect to be exerted by blood and blood serum, the former being more active in this respect although neither substance entirely abolishes the antibacterial action of the drug. Studies on the mode of action of the compound are now too incomplete to provide an adequate explanation for this phenomenon. Experience with other antibacterial substances suggests the possibility of antagonistic substances common to blood and certain bacteria which inhibit the activity of the compound, but it is also possible from evidence obtained from preliminary biochemical tests with the compound in blood that this effect may be due to chemical changes in the compound itself resulting in this modification of its action in the presence of blood.

Perhaps the most striking effect of the compound is its trypanocidal action. The fact that it is effective by the oral administration of a single dose is very rare among trypanocidal compounds, most of which must be given parenterally.

Furthermore, a calculation of the therapeutic index for mice using 50 per cent values $\left(\frac{\text{LD50}}{\text{CD50}}\right)$ gives a ratio of 9 for the compound, while for rats, calculation of $\left(\frac{\text{LD100}}{\text{CD100}}\right)$ results in a ratio of 8, indicating in both instances a satisfactory margin of safety between the therapeutic and toxic dose of the compound. In addition, much smaller doses are effective in removing trypanosomes from the blood of mice and rats and it is probable that repetition of these smaller quantities would prove to have a curative effect thereby further increasing the margin of safety for the compound.

Furacin in a suitable base has already been shown by Snyder, Kiehn and Christopherson (8), McCollough (9) and by Shipley (10) to be effective clinically in the treatment of infected wounds and surface lesions by local application. Further work is now being done on the tolerance and clinical effectiveness of the drug after oral administration.

It is also interesting to note that while an extension of the previous work to include several hundred compounds has further demonstrated the property of a nitro group in the 5-position to confer antibacterial activity *in vitro* on a variety of simple furan compounds an examination of approximately one-half of these compounds for a similar action *in vivo* following oral administration has shown only those closely related to the semicarbazone to be effective.

SUMMARY

1. 5-nitro-2-furaldehyde semicarbazone was shown to exert antibacterial action *in vitro* on a variety of Gram positive and Gram negative species of bacteria, also upon *M. tuberculosis*.

2. The LD50 of the compound following oral administration to mice was determined on seven samples. It was also found that the chief symptom of toxicity from either oral or parenteral use in rats and mice was hyperirritability.

3. Lethal doses of the compound produced no definite histopathology when fed to mice and rats, but subcutaneous injections of large doses produced marked changes in the structure of the liver and kidneys.

4. 5-nitro-2-furaldehyde semicarbazone was effective by oral administration in protecting mice infected with *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Salmonella schottmülleri* and *Salmonella aertrycke*. It was ineffective against pneumococcus infections.

5. The size of the infecting inoculum was shown to affect the survival rate obtained, especially with Gram negative species.

6. Single oral doses of the compound had a marked therapeutic effect on infections with *Trypanosoma equiperdum*.

7. The compound was not effective in the therapy of infections in rats with *Spirocheta novyi*. Preliminary experiments showed the compound to be active *in vitro* on *Treponema pallidum* and the dark-field findings and course of scrotal lesions in rabbits infected with this organism were modified.

8. The subcutaneous injection of 5-nitro-2-furaldehyde semicarbazone also

protected mice infected with *Streptococcus hemolyticus* and *Trypanosoma equipercum*.

9. 5-nitro-2-furaldehyde semicarbazone was also effective in the therapy of mice infected with *Streptococcus hemolyticus* when fed as a part of the daily ration.

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A BIOLOGICAL ASSAY METHOD FOR DETERMINING 2,2 BIS (p-CHLOROPHENYL)-1,1,1 TRICHLOROETHANE (DDT)[1]

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PRINCIPLE. Quantitative estimation of 2,2 bis (p-chlorophenyl)-1,1,1 trichloroethane or DDT depends on the toxic response of the common house fly, Musca domestica. This toxic response is characteristic for DDT and can be sharply differentiated from the effects of isomers or degradation products, including DDA (di (p-chlorophenyl) acetic acid) (1). When the insect walks over a glass surface upon which DDT has been deposited by evaporation of a solvent such as ether, characteristic symptoms supervene within one or two hours. The fly is so sensitive to DDT, that under proper conditions exposure of 100 individuals to a glass surface containing 5 micrograms causes about 50 per cent mortality. This LD50 is of the order of 0.05 microgram/20 mgm. fly or 2.5 mgm./kgm. of flies. Quantitation is made on the basis of the familiar dosage-mortality relationship which is established for 4 to 6 dosage levels.

CULTURE AND METHODS FOR HANDLING THE INSECTS. The methods evolved for this study have proved to be fairly convenient and useful. Better methods will undoubtedly suggest themselves to those versed in procedures in entomology. Ordinary house flies are placed in five-gallon museum jars containing a 2-inch layer of rabbit manure mixed with pieces of ground horse meat. During development of the maggots frequent additions of meat are made together with sufficient water to keep the breeding matrix moist. The jars are covered with copper screen and kept in a hood ventilated by an exhaust fan. A jar produces approximately 4000 flies. Six generations of flies have been bred from the original stock without the addition of "new blood." Within a few hours after a batch of flies has emerged from their puparia, they are transferred to a 10-liter capacity wire mesh cone. About 3000 flies can be thus housed until ready for assay. They are fed on sucrose solution. In general, most consistent and reproducible assays are made with flies used soon after emergence. Flies allowed to become older than a week usually give unreliable results, characterized chiefly by a rise in the mortality of the controls.

ISOLATION OF DDT FROM BIOLOGICAL MATERIAL. Extraction of DDT from tissues or excreta of animals poisoned with DDT is made with ether. Wet or dehydrated (Na_2SO_4) tissues are pulped with a small quantity of washed sea sand in a mortar. Successive 10-ml. portions of ether are added, thoroughly mixed with the tissue brei and decanted into a volumetric flask of appropriate size. By careful decantation perfectly clear ether extracts can be prepared which require no filtering. In the case of fluids such as blood, urine or bile, successive ether extractions are made in separatory funnels. Emulsions can usually be abated with a few drops of absolute alcohol. On the basis of preliminary orienting tests, an aliquot of the ether solution containing 2.5 to 10 micrograms of DDT is evaporated in the test flask. The residue after evaporation of the ether will contain besides DDT varying amounts of fat or other ether-soluble tissue extractives. Excessive amounts of these residues have been found to interfere with the assay by contributing substances which are toxic to flies. In practice, it has been found that ether extract residues from more than 0.5 to 1.0 gram of tissue, particularly liver and kidney, make the DDT assay unreliable. This, in effect sets the lower limits of DDT determinable in these tissues at about 2.5 p.p.m. Fortunately, the toxic effects of tissue extractives on the fly can be sharply differentiated from those of DDT: "Tissue toxicity" in the fly is slow in onset and produces torpidity; DDT toxicity occurs early and produces an excitatory reaction. By means of this expedient it has frequently been possible to gauge roughly amounts of DDT smaller than 2.5 p.p.m. in tissues.

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[1] A portion of the funds used in this investigation was supplied by a transfer, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Division of Pharmacology of the Food and Drug Administration.

ASSAY PROCEDURE. The flies are exposed to DDT in a wide-mouthed Erlenmeyer "beaker flask" of 250-ml. capacity. This test vessel has straight sides forming an acute angle with the base, which is a flat surface of about 40 $(\text{cm.})^2$ area. Upon this surface DDT is deposited by evaporation of an ether solution containing either the standard or the unknown. A gentle jet of filtered air is used to drive off the ether, heat being avoided. Aqueous residues resulting from condensation of water must also be completely removed. One test flask is prepared for each unknown; 10 to 20 such samples can be run conveniently at one time. With each group a series of 4 to 6 test flasks containing known amounts of DDT must be included for construction of the standard assay line. After the test vessels have been prepared with DDT residues they are weighed to the nearest 0.01 gram and are then ready to receive the flies. One hundred flies are transferred to each flask by counting them as they file singly through a small hole in a celluloid funnel. This funnel is connected to a wire mesh storage cone, capable of containing approximately 3000 flies without undue crowding. The test flask with its 100 flies is quickly capped with a perforated filter paper and again weighed. The flies are then allowed to remain in the flasks overnight for about 20 hours. At the end of this time the mortality ratio is obtained by separate count of the living and dead insects. All flies which are still living, but too helpless to walk or fly (about 10 per cent) are arbitrarily counted as dead.

CALCULATIONS. When the standard doses of DDT, expressed as mgm./kgm. of flies are plotted against the corresponding mortality ratios, the points will determine a sigmoidal dosage-mortality curve. However, when the dosage is expressed in logarithms, and the per cent mortality in probits, according to the method of Bliss (2), the sigmoidal curve is converted to a rectilinear one. In each assay this relation between "probit kill" and log-dose is determined by 4 to 6 points which lie approximately in a straight line. The best straight line through the points is drawn on the basis of visual inspection. For additional refinement the line may also be calculated by the method of least squares. This, however, is probably not justified in view of other errors which may be of more consequence. Unknown amounts of DDT are calculated graphically by reference to the standard assay line which must be constructed for each set of determinations.

CONDITIONS AFFECTING THE DOSAGE-MORTALITY LINE. With the exception of the sex ratio to be discussed later, the physical conditions under which standard and unknown were run were identical. The conditions adopted for the assay were as follows: 100 flies were exposed to DDT residue for a 20-hour period beginning in midafternoon and ending in midmorning of the following day. Depending on the season of the year, exposures were approximately 8 to 10 hours in natural light and 10 to 12 hours in darkness. During the summer the flies were exposed in a constant temperature room at 21°C. and in the winter at room temperature 25°C. ± 1°C. With the exception of urine all DDT residues were in a dry condition when the flies were brought into contact. The sensitivity of this assay, as reflected in changes of the LD50 and slope of the dosage-mortality line, was significantly affected by variations in the physical conditions. Some of these are to be considered below.

(a) *Rate of uptake of DDT by the fly.* Batches of 100 flies were exposed to 10-microgram residues of DDT for ½, 1, 2, and 4 hours, at the end of which time they were transferred to clean flasks and the mortality ratios determined after 20 hours. When the mortality ratio was plotted against the time of contact, the relationship was best described by a sigmoidal curve, as shown in figure 1. Exposure of a fresh batch of flies to each of the residues showed that the "2 and 4 hours flies" had removed all of the DDT, while the "½ and 1 hour flies" had left sufficient DDT to produce mortality ratios of 47 and 14 per cent respectively. Complete recovery of all of the DDT was precluded by the limitations on the sensitivity of the method.

(b) *Weight of the fly.* In the course of application of this method to biological materials, a total of 39 assays were run. For each assay a dosage-mortality line was laid off through 4 to 6 points. Such representative lines can be seen in figure 2. It soon became apparent that considerable variations in the parameters defining the dosage-mortality line occurred from one assay to the next. Thus, for the slope the extreme range was 2.4 to 6.8 probits per log dose; for the LD50, 1.3 to 3.8 mgm./kgm. Plotting the average body weight of the flies in each batch against the corresponding LD50 of each dosage-mortality line showed a significant positive correlation between them (r = +0.88). The equation describing the relationship, as calculated from the data of 39 assays, is as follows:

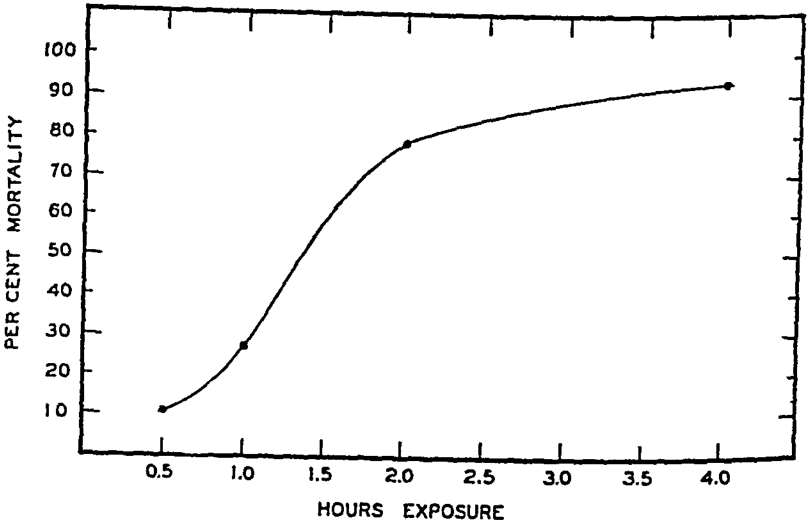

Fig. 1. The Rate of Uptake of DDT by the Fly

$$LD50 = 0.000038 \times (\text{weight})^{2.4}$$

where LD50 is expressed in micrograms and the weight of the fly in milligrams. Stated in another way this means that more DDT is required to kill a kilogram of "heavy" flies than it does a kilogram of "light" flies. Similarly, plotting the average body weight of the flies in each batch against the corresponding slope of each dosage-mortality line, there was shown to be a significant positive correlation (r = +0.40). Without describing the relationship by an equation, it can be stated that this indicates that as the average body weights of the flies in a batch tend to increase, the slopes of the corresponding dosage-mortality lines tend to become steeper.

(c) *Length of the assay period.* While it has been shown that flies take up all of a toxic dose of pure DDT residue within 4 hours, there is a lag in the appearance of toxic symptoms in many of the insects. Furthermore, it has been shown that the presence of tissue extractives frequently delayed the uptake of DDT. For these reasons all exposures were made over a 20-hour period. Nevertheless, because of the delayed "tissue toxicity" that occurred after 10 to 15 hours, it seemed desirable to explore the possibility of circumventing this effect by shorter exposures. It was found that the advantage gained by reduction of the toxic tissue effect was offset by loss in effective DDT toxicity. For example, the LD50 of a 7-hour assay curve was 4.6 mgm./kgm. as compared with the LD50 of 2.6 mgm./kgm. of a 20-hour assay curve. See figure 2, C.

(d) *Moisture.* Addition of small quantities of moisture to a DDT residue shortened the toxic induction period and produced a very measurable decrease in the LD50. See figure 2, D. With the exception of the urine this technique was not adopted in the assay procedure because when tissue residues were present, moisture seemed to enhance the "toxic tissue effect." In the case of urine, however, it was found that dry residues in some way interfered with the DDT getting in contact with the feet of the fly. Only by addition of 0.1 ml. water to each urine residue was it possible to recover added DDT.

(e) *Temperature.* Lowered temperature made the flies much more sensitive to DDT. While all winter assays were run at 25°C. ± 1°C., the high summer temperatures of 32°C. and higher made it necessary to run all assays during this period in a constant-temperature room set at 21°C. See figure 2, E.

(f) *The effect of light.* Natural light as compared to darkness made the flies more sensitive to DDT. It is presumed that the effect of light is not specific, but simply keeps the insects stimulated and thus more liable to frequent contact with the DDT. See figure 2, F.

(g) *Sex ratio.* The sex ratio of the flies bred for these experiments tended to be unity. It is well-known, however, that segregation of the sexes tends to occur when the insects are permitted to choose a path of egress from a container. Thus, when a full storage cone was gradually emptied by counting out groups of 100 flies into a series of say 30 test flasks, it was found that the sex ratio might favor males over females by 7 to 3 in the first 10 flasks, but tended to favor females over males in the last 10 flasks. In Fig. 2, G are shown two dosage-mortality curves in which there was a marked difference between the sex ratios. Since the average male fly is lighter than the female, it is to be expected that the LD50 for the 75/25 ratio batch would be lower. This is in keeping with the observations made earlier in the paper. These data should not be taken as excluding the possibility that we are dealing here also with an intrinsic sex factor which is independent of weight. It is recognized that this type of variable condition which obtains unequally for standard and unknown may help to explain some of the erratic results encountered occasionally. Certain obvious means of mitigating this source of error have been tried, such as taking as reference an average dosage-mortality line constructed from two dosage-mortality lines, one at the beginning and one at the end of each assay.

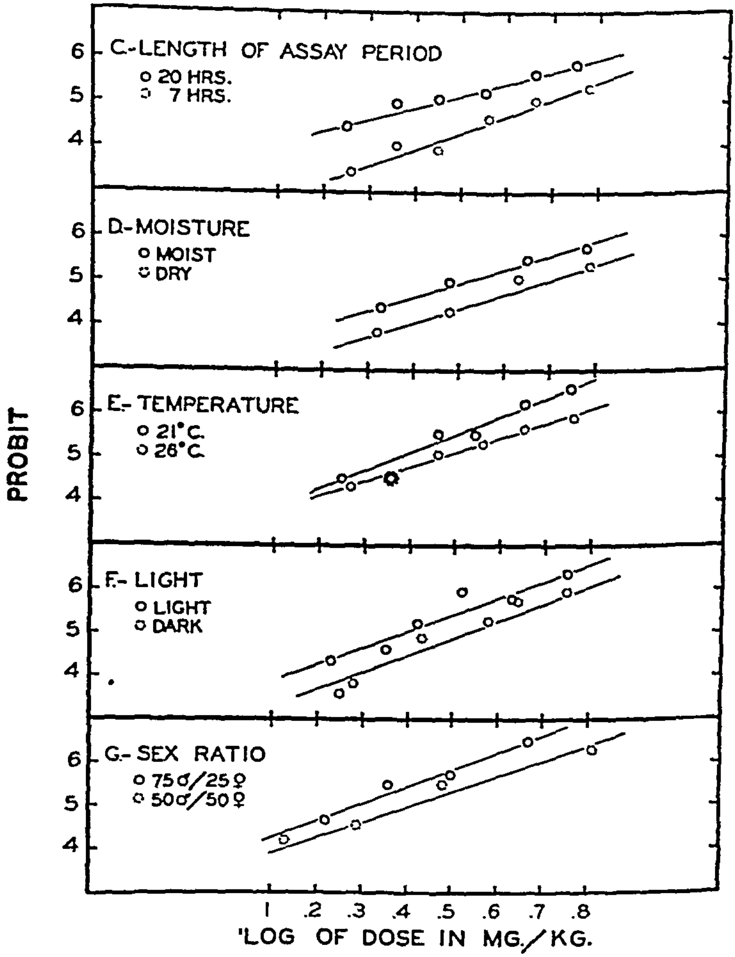

FIG. 2. CONDITIONS AFFECTING THE DOSAGE-MORTALITY LINE

(h) *The Blank, or control mortality ratio.* In all assays, several control flasks of flies were carried through the 20-hour period in order to determine the behavior of the insects. If the insects were "young," properly fed and watered there were usually no deaths among a group of 100 flies. Occasionally mortalities of as high as 6 per cent were observed, but when they reached 10 per cent or more there was reason to suspect the reliability of the entire assay.

TABLE 1

The LD50 of DDT and related compounds for Musca domestica

COMPOUND	LD50
	mgm /kgm.
DDT	
2,2 bis (p-chlorophenyl)-1,1,1 trichloroethane..	2.23 (average 39 assays)
DDA	
di (p-chlorophenyl) acetic acid..	500 (greater than)
2,2 bis (p-chlorophenyl) 1,1 dichloroethylene	500 (greater than)
di (p-chlorophenyl) ketone . ..	500 (greater than)
p-chlorobenzoic acid . . .	500 (greater than)
DDT (o,p,isomer)	
2,2 bis (o,p chlorophenyl) 1,1,1 trichloroethane.	35–55
DDD	
2,2 bis (p-chlorophenyl) 1,1 dichloroethane	21
Gammexane	
Gamma hexachlorobenzene .	0.5–0.7

TABLE 2

A comparison between the chemical and biological method for determining DDT

RAT NO	SCHECHTER COLORIMETRIC*	BIOLOGICAL ASSAY	
		Assay 1	Assay 2
	Mgm. DDT per gram perirenal fat from animals fed DDT		
1	3.0	3.7	2.0
2	3.9	4.4	5.3
3	2.2	2.2	2.0
4	3.2	3.8	2.8
5	2.5	2.0	2.2
6	3.2	2.6	2.8
	Mgm. DDT per 100 ml. DDT solution subjected to ultraviolet deterioration		
	356	445	540
	743	640	751
	641	377	469
	1320	1105	1300
	2830	1764	2280

* These analyses were made by Mr. P. A. Clifford, Food Division.

THE TOXICITY OF DDT AND RELATED COMPOUNDS. In table 1 are given the LD50's of a number of substances, some of which could possibly occur as degradation products of DDT in the organism. Obviously this assay method for DDT would prove useless if any of them had toxicities of the same order as DDT. From the results it can be seen that none interfere since they all have a toxicity of a different order. It should be noted that DDA, a proved metabolite of DDT is practically nontoxic. Because of the difference between the p,p and o,p isomers of DDT (the latter being a frequent contaminant of the commercial product) this assay method suggests a means of assessing the purity of DDT preparations.

TABLE 3

The recovery of DDT added to rabbit and rat tissues and rabbit urine

SUBSTANCE	AMOUNT	DDT ADDED MICROGRAMS	DDT RECOVERED MICROGRAMS
Urine	20 ml	7.7	7.2
Urine	20 ml	7.7	6.2
Blood	5 ml	10.0	7.8
Kidney	4.3 gms	10.0	10.1
Liver	4.8 gms	10.0	10.7
Liver	3.6 gms	100	89
Kidney	0.66 gms	100	105
Liver	2.88 gms	100	84
Kidney	0.59 gms	100	99
Liver	8.61 gms	100	91
Kidney	2.19 gms	50	50
Liver	5.04 gms	50	44
Kidney	2.71 gms	25	21
Liver	8.47 gms	100	64
Liver	6.32 gms.	50	52
Kidney	2.57 gms	25	13
Liver	5.83 gms	25	12
Spleen	0.55 gms	20	21
Spleen	0.56 gms	25	22
Liver	2.09 gms	100	170, 137
Muscle	1.00 gms	25	26
Spleen	0.85 gms	25	32

Gammexane, of course, leads the list in toxicity and could not be differentiated by this method if it were mixed with DDT.

RESULTS. The biological assay method was compared with the Schechter colorimetric method (3). Some results are presented in table 2. The bio-assays were done in duplicate, using different batches of flies. In the case of DDT in perirenal fat, the two methods show essential agreement. In the case of the deterioration samples, the chemical method tended to give somewhat higher results.

In table 3 are given some data on the recovery of DDT added to urine and tissue. While these experiments, of course, do not decide whether it is possible to extract completely DDT residing in tissues of poisoned animals, they do show that within the limits of a biological assay method added DDT is recoverable. In general, there is a tendency for recoveries to be somewhat low. No explanation can be offered for the occasionally extremely high or low recoveries.

SUMMARY AND CONCLUSIONS

A biological assay method depending on the toxic response of the house fly has been developed to determine DDT in animal tissues and excreta. Quantities of DDT of the order of 2.5 p.p.m. can be determined.

The author wishes to thank Dr. B. J. Vos of this Division for help in the statistical analyses, and Mr. P. A. Clifford, Food Division, for performing the chemical analysis of DDT by the Schechter method.

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2,2-BIS (p-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE (DDT) IN THE TISSUES, BODY FLUIDS AND EXCRETA OF THE RABBIT FOLLOWING ORAL ADMINISTRATION[1]

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Recent studies on the effects of DDT in mammals have treated largely of the toxic response of the organism (1, 2) and of the pathological changes induced in tissues (3, 4). Comparatively little unchallenged information is available on the concentration of DDT in the tissues and excreta of animals following acute and chronic exposure (5, 6). Smith and Stohlman (2) on the basis of their organic chloride method (7), which is not specific for DDT alone, claim to have found measurable quantities of DDT in the tissues and excreta of cats and rabbits. This, however, could not be confirmed in the case of rabbit urine by means of X-ray diffraction analyses (5). Again, in a somewhat later publication, Stohlman (8) has reported the isolation and identification of crystalline DDT from the urine of DDT-poisoned rabbits. Since the urine was collected from the cages, instead of from the bladder by catheter, it may be seriously questioned whether the DDT found did not come from feces by leaching action of the urine. Recently Stiff and Castillo (9) using a xanthydrol reaction for determining DDT (10) were unable to find detectable amounts of DDT as such in the organs and body fluids of acutely poisoned rabbits, although they did confirm the presence of organic chloride which Smith and Stohlman have assumed to be DDT. It seems, therefore, desirable to report at this time the analyses of tissues and excreta for DDT by a more specific and sensitive method.

METHOD. Rabbits were given a 10 per cent solution of DDT in corn oil by stomach tube. From 200 to 400 mgm./kgm. were given in a single dose and this produced characteristic tremors in about half of the animals, irrespective of the state of fullness of the alimentary tract. In a series of animals used for tissue analyses, several received the stated dose daily for a period of 2 to 4 days. Most of these animals showed severe tremors. Ether extracts were prepared of tissues, body fluids and excreta and DDT was determined by a bio-assay method (11) based on the toxic response of the house fly. Check analyses run by the Schechter and Haller (12) method showed that the two methods were in essential agreement. Furthermore, it was shown that neither the o,p isomer of DDT, nor the degradation products, such as the ketone and the dehydrochloride, nor di (p-chlorophenyl) acetic acid (5), recently isolated from rabbit urine interfered, because their toxicities for the fly were found to be comparatively low. The lower limits for quantitatively determining DDT by the bio-assay method were of the following order: For tissues, blood, feces, bile, 2 to 5 micrograms per gram; for urine, 5 micrograms per 20 milliliters. Attempts to determine smaller concentrations of DDT by taking larger samples were met by a type of interference characterized by a toxic reaction of the test insect which was not typical of DDT.

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[1] A portion of the funds used in this investigation was supplied by a transfer, recommended by the Committee of Medical Research, between the Office of Scientific Development and the Division of Pharmacology of the Food and Drug Administration.

RESULTS. A. *Feces.* From table 1 it can be seen that enormous quantities of DDT are excreted in the feces. This may be presumed to be simply unabsorbed DDT, and possibly explains the extreme variability of rabbits in their toxic response following oral doses of DDT. The data also indicate, as might be expected, a lag in excretion. Even allowing for the extreme insolubility of DDT, it would seem quite probable that leaching of the feces by urine would render DDT analyses of cage urine unreliable. In fact some analyses of cage urine were made; they showed varying amounts of DDT ranging from 5 to 50 micrograms per 20 ml. sample.

TABLE 1

The excretion of DDT in the feces of rabbits following oral administration of a single dose of 350 mgm./kgm. in corn oil

RABBIT NO.	MICROGRAMS DDT PER GRAM FECES				
	Time in hours after dose				
	20	44	51	71	93
1	1280	21000	1370	717	81
2	21800		5200	223	17
3	295		1450	283	50

TABLE 2

Recovery of DDT added to rabbit urine

AMOUNT OF URINE	DDT ADDED	DDT FOUND
ml.	*micrograms*	*micrograms*
20	10.0	11.7
20	7.0	6.2
20	5.0	4.5
20	10.0	12.0
20	10.0	11.5
20	10.0	11.0

B. *Urine.* In an attempt to decide whether DDT is excreted in the urine of the rabbit, catheter specimens were taken from a series of 12 animals at various intervals starting 3½ hours and extending to 72 hours after a single dose of DDT. Within the limitations of the method in no instance could DDT be demonstrated in any 20 ml. sample of catheter urine. The possibility that certain ether extractable residues from urine might occlude DDT and so hinder its absorption by the fly was investigated. However, in a large number of samples where DDT was added to urine, it was possible in every case to recover the added DDT. Representative examples of this are shown in table 2.

Considering the alkalinity and the rapid putrefactive changes which occur in rabbit urine, there was also the possibility that DDT might be destroyed before analysis could be made. This was tested by adding small quantities of DDT to 20 ml. portions of urine and allowing the mixtures to incubate for varying periods of time. Table 3 shows the results. There is no trend indicating disappearance of DDT with time.

TABLE 3

Incubation of DDT with rabbit urine

AMOUNT OF URINE	DDT ADDED	DDT FOUND	PERIOD OF INCUBATION
	Rabbit No. 1		
ml.	*micrograms*	*micrograms*	*hours*
20	7.0	7.1	0
20	7.0	5.8	0.5
20	7.0	5.6	1
20	7.0	6.1	19
	Rabbit No. 2		
20	7.0	6.2	0
20	7.0	7.1	0.75
20	7.0	5.1	19

TABLE 4

The DDT content of rabbit tissues and fluids following single and repeated daily doses of DDT dissolved in corn oil

RABBIT NO.	MGM. DDT PER KGM. RABBIT IN SUCCESSIVE DAILY DOSES				TOXIC RESPONSE	MICROGRAMS DDT PER GRAM OF FRESH TISSUE OR MILLILITER OF FLUID					
	1	2	3	4		Liver	Kidney	Brain	Blood	Urine	*Bile*
3744	350				Slight tremors	0.0	2.0	0.0	1.5	0.0	0.0
3753	350				Slight tremors	0.0	1.7	0.0	1.1		
3741*	350	200			Moderate tremors	3.5	10		1.9	0.0	15
3754	350	200			Moderate tremors	22	11		3.1	0.0	0.0
3742	350	200	200	400	Moderate tremors	10	20	16	3.6		13
3887	400				Severe tremors	12	13		8.0	0.0	29
3888	400	400			Convulsions, severe tremors		10		2.2		4.1
3897†	400	400	400		Moderate tremors	14	14		3.2		
3889	400	400	400		No tremors, diarrhea	6.4	7.6		7.5		0.0
3756‡	350	200	200	400	Slight tremors	2.0	4.4		1.1		

* Spleen sample contained 16 micrograms per gram.

† Fat sample contained 305 micrograms per gram; lean muscle, 15 micrograms per gram.

‡ With the exception of 3756, which was killed 11 days after last dose, all animals were killed 24 hours after their last dose.

C. *Tissues and body fluids.* In table 4 are given the results of tissue and body fluid analyses for DDT. In general, it can be seen that the animals which received the largest quantity of DDT gave the highest tissue concentrations. In the case of Rabbit 3889, it is presumed that diarrhea reduced absorption of DDT, and this may explain the lack of toxic response as well as the somewhat lower tissue levels of DDT. It is interesting to note that Rabbit 3756, killed 11 days after its last exposure to DDT, still retained small amounts in its tissues. Only one sample of fat was analyzed, but the concentration of DDT found therein was nearly 25 times greater than in the other tissues.

DISCUSSION. The failure of Stiff and Castillo (8) to find DDT in the body fluids and tissues may be ascribed to lack of adequate sensitivity of their method. On the other hand, where relatively large amounts of DDT are involved, as in feces, their value of 5.6 mgm. per gram is about of the same order of magnitude as reported in table 1 of this paper.

In general, Smith and Stohlman's (2) DDT concentrations in rabbit tissues are difficult to compare with our own, chiefly because there is much divergence between the number and size of the doses. However, in one instance 5 hours after administering 500 mgm./kgm. of DDT in olive oil, these authors report a blood value of 4.0 mgm. per 100 ml. (40 micrograms per ml.) and a similar value 26 hours after a 300 mgm./kgm. dose. This is from 5 to 40 times the amount shown in table 4, and under conditions roughly similar with regard to dose and time of assay.

In contrast to our failure to find any DDT at all in catheter samples of urine, Smith and Stohlman have reported concentrations ranging as high as 65 mgm. per 100 ml. This is most plausibly explained by the authors' assumption that all of the organic chlorides excreted in urine are DDT, a position no longer believed tenable in view of the more specific means for determining this substance.

SUMMARY

1. By means of a bio-assay method DDT has been found in tissues, body fluids and feces of rabbits acutely poisoned by oral doses of DDT in oil.

2. Within the limitations of this method no DDT could be found in the urine of such poisoned animals.

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TOXICITY OF CERTAIN HALOGEN SUBSTITUTED ALIPHATIC ACIDS FOR WHITE MICE[1]

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Several of the halogen-substituted aliphatic acids are potent choleretic agents (1, 2, 3). Some of them are also effective in inhibiting alcoholic fermentation and in retarding or inhibiting the metabolic processes of various molds, yeasts and bacteria (4, 5, 6). Since such properties make these compounds of interest therapeutically and also make them of potential use in food processing it is essential to know something of their toxicity.

The literature contains few references to the toxicity of any of the halogenated fatty acids except the three chlorine-substituted acetic acids and iodoacetic acid. It was considered desirable, therefore, to determine the acute toxicity of other available compounds of this type.

METHOD. The pH of a 5 per cent aqueous solution of the compounds studied varied from 0.5 to 2.00. Preliminary tests disclosed a local irritating factor in the effects. To avoid this the 5 per cent solutions of the acids were neutralized (pH 7.0) with sodium carbonate, and used at once since it has been shown (7) that solutions of the sodium salts of many of the compounds hydrolyze rather rapidly.

Adult white mice, fasted for 24 hours previously, were used throughout as the test animal. To groups of 30 animals varying volumes of each of the neutralized halogenated acid solutions were administered by way of the esophagus with a tuberculin syringe and a large bore blunt hypodermic needle. All animals which died within 5 days were considered, arbitrarily, to have been killed by the compound since no deaths occurred among a large group of control animals from the same litters. From the accumulated data toxicity curves were plotted and the LD_{50} for each agent was estimated. The estimated LD_{50} for the various compounds is listed in Table 1 both in milligrams per kilogram and in millimols per kilogram.

DISCUSSION. Note that the toxicity of these compounds decreases as the length of the carbon chain increases. These results are contrary to Richardson's observations in regard to the toxicity of alcohols, which seemed to indicate that as the carbon chain lengthened the toxicity increased (8).

The stomach capacity of a mouse was found to be 1 to 2 cc. at most, therefore the LD50 of beta-chloropropionic, beta-bromopropionic, alpha-bromoisobutyric and alpha-bromoisovaleric acids could not be determined using 5 per cent solutions. Since doses of 2 grams per kilogram killed no mice the LD50 is reported as greater than 2,000 mgm. for these compounds.

Substitution of a halogen molecule for one of the hydrogen molecules in the alkyl radical of a fatty acid produces marked changes in the chemical-physical reactions of the acid (4). Changes in dissociation, in solubility and in activity occur. No less marked changes appear in the physiologic action of the fatty acids following such a substitution. It has been shown previously (4) that monochloroacetic, monobromoacetic and monoiodoacetic acids inhibit gas production of yeast more readily than acetic acid and that this property becomes more pronounced as the molecular weight of the halogen is increased. The present experiment indicates that this is also the case when the death of mice is used as a criterion for comparison since iodoacetic acid is more toxic than bromoacetic which in turn is more toxic than chloroacetic acid. In those cases in which homologous compounds were used the bromine containing compound was always more toxic than the chlorine homolog.

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[1] A portion of this work was completed in the Pharmacology Laboratory of West Virginia University.

The position of the halogen in the chain apparently is important in the physiologic action of the chemical agent. Although the beta substituted compounds are more easily hydrolyzed at pH 7, hydrolysis is probably too slow to account entirely for their lower toxicity. The beta compounds have lower oil/water solubility coefficients than their respective alpha homologs which may influence their action by preventing ready absorption through the gastro-intestinal mucosa or penetration of cells and tissues. Hoffman, Schweitzer and Dalby (9) state that biologic activity of an acid is dependent on its dissociation and that the less ionized the acid the more powerful its biologic effect. This does not seem to apply to the toxicity of the halogenated fatty acids since the least dissociated acids are least toxic.

TABLE 1

The oral LD50 for mice of 5 per cent solutions of halogen substituted fatty acids neutralized to pH 7

ACID	LD50 IN MGM./KGM. BODY WEIGHT	CALCULATED LD50 IN MM/KGM. BODY WEIGHT
Monochloroacetic	165	1.73
α-Chloropropionic	980	9.00
β-Chloropropionic	over 2,000	
Iodoacetic	83	0.44
Monobromacetic	100	0.72
α-Bromopropionic	250	1.63
β-Bromopropionic	over 2,000	
α-Bromobutyric	310	1.86
α-Bromoisobutyric	over 2,000	
α-Bromovaleric	380	2.65
α-Bromoisovaleric	over 2,000	
α-Bromo-n-caproic	590	3.02

SUMMARY

The oral toxicity for mice of eight halogenated fatty acids increases in relation to the increase in molecular weight of the substituted halogen and decreases as the carbon chain is lengthened. In the case of four acids the LD50 of which could not be readily determined, substitution of halogen in the beta position markedly reduces the toxicity as does branching of the carbon chain.

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THE BIOLOGICAL ASSAY OF EPINEPHRINE[1]

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This study deals with the application of both the experimental design proposed by Bliss and Marks (1, 2) for the assay of insulin and the chart and nomograph described for the assay of penicillin by Knudsen and Randall (3) to the canine blood pressure method for the biological assay of epinephrine, one version of which is currently official (4). It appeared probable that the desirable objectives to be attained through such an application might include (a) the utilization of all of the data obtained in an assay in reaching an accurate, objective estimate of potency; (b) a relatively easy method of computing the reliability of individual assays; and (c) an over-all procedure sufficiently simple and economical of time to be practical for routine work.

One of the disadvantages of the present official method is the failure to satisfy fully the first objective cited above. Although extended comparisons of the standard and the unknown may yield an accurate estimate of potency, the method fails to provide for the utilization of all of the data obtained and is therefore inefficient. Other disadvantages have been discussed by Thompson (5).

Thompson (5), utilizing the experimental design and method of analysis of data proposed for ergonovine by Vos (6), recently proposed a method for the assay of epinephrine which yields assays of a degree of accuracy seldom attained by means of a biological assay procedure and which seems to be an ideal tool for use where a need for extreme accuracy outweighs other considerations. However, the method of analysis of data upon which the procedure is based is laborious.[2]

The dose-response relationship of epinephrine in the dog has been studied by Hjort, deBeer, and Randall (7). In the present study this relationship was assumed to be valid and no further detailed study of it was undertaken.

Experimental. The assay procedure as finally developed is as follows: A healthy dog.[3] is selected as directed for the method now official (4), anesthetized by means of the intraperitoneal injection of 150 milligrams per kilogram of Na phenobarbital, atropinized as in the U.S.P. XII method, and arranged for the recording of the carotid blood pressure by means of the conventional Hg manometer. A dose of U.S.P. Reference Standard Epinephrine solution, usually diluted 1–50,000 or 1–100,000, which gives a blood pressure rise of approximately forty mm. Hg is selected. This dose is designated the low dose of the standard (s_L), and 1.5 times this volume the high dose of the standard (s_H). The unknown solution is diluted on the basis of available data (label, manufacturing information, etc.) so that it is theoretically equal in potency to the diluted standard solution. A volume of the diluted unknown solution equal to s_L is designated the low dose of the unknown, u_L, and 1.5 times this volume is the high dose of the unknown, u_H. Each of these four doses is administered four times in order determined by assigning the various doses at random to a 4 x 4 latin square (1), an example of which is shown in table 1. The injections are made into the saphenous vein at five-minute intervals. Each of the sixteen responses as indicated by the rise in blood pressure is measured to the nearest millimeter.

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[1] A preliminary note covering part of this work was published in Federation Proceedings, 3: 80, 1914

[2] Since this article was written, a paper by R. H. Noel on the same subject has appeared (This Journal, 84: 278, 1945.) His article uses a similar experimental design but a different method of computing the potency and standard error of assay. The present treatment is believed to offer the advantage of speed and simplicity. Control chart technics for use with this approach are described by Knudsen and Randall (3).

[3] Dogs which failed to meet the sensitivity requirements of the U.S.P. XII were not used in this study

TABLE 1

Typical dosage schedule

	FIRST DOSE	SECOND DOSE	THIRD DOSE	FOURTH DOSE
First group	u_L	s_H	s_L	u_H
Second group	s_H	s_L	u_H	u_L
Third group	s_L	u_H	u_L	s_H
Fourth group	u_H	u_L	s_H	s_L

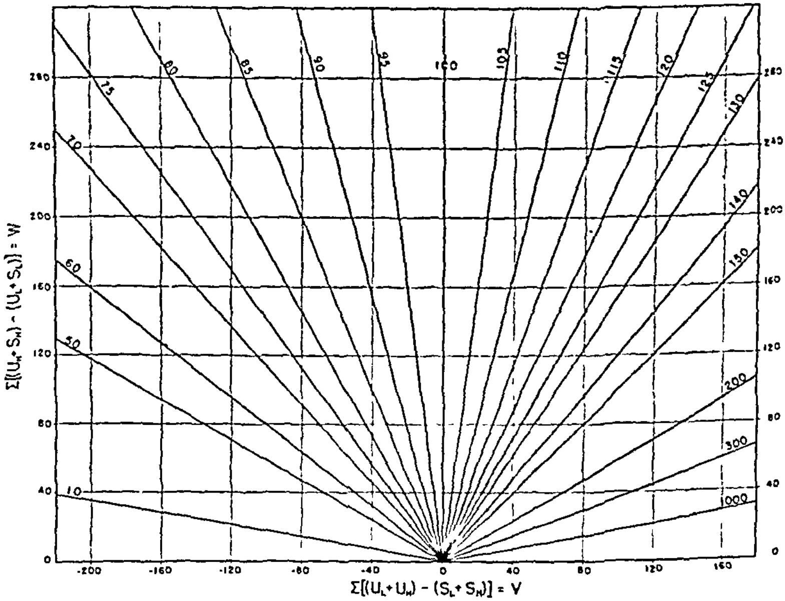

FIG. 1. CHART FOR DETERMINING POTENCY WHEN THE RATIO OF THE HIGH DOSE TO THE LOW DOSE IS 1.5 TO 1

The data thus obtained may be treated in either of two ways in order to extract essentially identical estimates of the potency and of the standard error of the assay. One method is to proceed by machine calculation as indicated by Smith and Vos (8) for the biological assay of posterior pituitary solution. *If this method is used, the calculations of the potency* and of the standard error are made exactly as in the pituitary assay except that in the case of the epinephrine assay the logarithm of the ratio of the high dose to the low dose is 0.176, and this value is substituted for the comparable figure 0.301 used in the pituitary assay.

A much simpler way of estimating the potency of the unknown and determining the standard error of the assay is by means of the chart and nomograph shown in figures 1 and 2 respectively. In this, four values, V, W, R_v, and R_w, obtained from the experimental data by means of addition and subtraction are used as follows[4]:

According to the design, the assay consists of four groups of four doses each, wherein each group consists of s_H, s_L, u_H, and u_L though not necessarily in that order. For each group of four doses

$$v = (u_L + u_H) - (s_L + s_H)$$

and

$$w = (u_H + s_H) - (u_L + s_L) \text{ are calculated.}$$

Then the four values mentioned above as necessary for use of the chart and nomograph are obtained from these values of v and w. The sum of the v's (or Σv) equals V and the sum of the w's (or Σw) equals W. The difference between the highest and lowest value of v equals R_v (the range of the v's) and the difference between the highest and lowest value of w equals R_w. The four responses to each dose should also be totaled and V and W should be checked by performing on these totals the same additions and subtractions used for v and w.

TABLE 2

Calculation of data

	u_L	u_H	s_L	s_H	$v = (u_L + u_H) - (s_L + s_H)$	$w = (u_H + s_H) - (u_L + s_L)$
Group 1 ...	40	46	40	62	−16	28
Group 2	38	39	42	58	−23	17
Group 3 ..	35	40	40	60	−25	25
Group 4 .	35	41	42	56	−22	20
Totals	148	166	164	236	$V = -86$ $R_v = 9$	$W = 90$ $R_w = 11$

Potency = 68
Standard error of assay = 4.1

The potency is determined by entering the chart (fig. 1) with the values of V and W and reading the potency from the radial lines.

The standard error of the assay is obtained from the nomograph (fig. 2) by using V, W, R_v, R_w and the potency. On the right-hand side of the nomograph connect the scale values of V and W with a straightedge. Mark with a sharp pointed pencil the point of intersection of this straightedge with the diagonal line. Then connect this marked point of intersection with the value of R_w and read the value of Q. Add Q^2 and R_v^2 obtained from the table of squares in the center of the nomograph to secure the value of T. On the left-hand side of the nomograph connect with a straightedge the values of T and W and read the value of the ratio from its scale. Multiply this ratio by the potency previously obtained and the result is the standard error of the assay.

An example may clarify the procedure. Table 2 gives the 16 blood pressure responses obtained in an assay and the simple calculations made on those responses.

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[4] The mathematical derivation of the method using the chart and nomograph is the same as that given by Knudsen and Randall for the penicillin assay (3).

Find the point on the potency chart corresponding to the values of $V = -86$ and $W = 90$. This point is nearest the radial line labeled 68 on the enlarged chart 5. Therefore, the potency is 68 per cent of the standard. To calculate the error of the assay from the nomograph[5] connect with a straightedge the values of $V = 86$ (for this step the sign of V is disregarded) and $W = 90$, and mark the intersection on the diagonal line. Then connect this marked intersection with $R_w = 11$ and read the value of Q which is 10.5. Now obtain

$$T = R_w^2 + Q^2 = 81 + 110 = 191.$$

On the left-hand side of the nomograph connect with a straightedge $T = 191$ and $W = 90$. The ratio is then read from its scale. In this case it is 0.06. When this ratio is multiplied by the potency (0.06 times 68) the standard error of the assay is found to be 4.1 per cent.

The value of W will be greater than twice the value of R_w if the slope of the dosage-response curve for that particular assay differs significantly from zero (by Student's "t" test). If W is less than twice R_w the assay is invalid and must be repeated.

TABLE 3

Assays of solutions of known potency

	TRUE POTENCY	MACHINE CALCULATION			FROM CHART AND NOMOGRAPH		
		Estimated potency	Std. error	Actual error*	Estimated potency	Std. error	Actual error*
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Assay 1 (8/18)	140	144.8	4.0	3.4	144.7	3.9	3.4
Assay 2 (8/11)	125	135.9	3.7	8.7	136.0	3.4	8.8
Assay 3 (1/6)	116	122.8	4.7	5.9	122.8	4.4	5.9
Assay 4 (4/21).	118	123.5	4.0	4.7	123.5	4.6	4.7
Assay 5 (4/27)	76	77.6	4.2	2.1	77.6	5.0	2.1
Assay 6 (12/30)	80	79.6	5.7	0.5	79.7	7.0	0.4
Assay 7 (5/7)	70	67.9	3.6	3.0	68.0	4.1	2.9
Assay 8 (2/10)	55	63.1	4.4	14.7	63.0	4.3	14.5
Assay 9 (6/30)	60	61.5	5.5	2.5	61.5	7.3	2.5
Mean . .			4.5†	5.1		5.1†	5.0

* $\frac{\text{Difference between true potency and estimated potency}}{\text{True potency}}$

† *Square root of the mean squares.*

The potency chart should be used only between 50 and 150 per cent for assay purposes. The radial lines indicating potencies beyond these limits are given for estimation purposes only.

Nine solutions of known potency have been assayed and the data treated by both of the methods described above. The results are presented in table 3. The solutions were prepared by diluting a solution of U.S.P. Reference Standard epinephrine, and the assayist (WTM) was never aware of the true potencies of the solutions under test until the results of the assay had been calculated.

DISCUSSION. If the data obtained in an assay of the design proposed are treated by machine calculation, the method has no advantages over that of Thompson (5) except a somewhat less complicated calculation procedure. However, when the chart and nomograph are used in making the necessary calcula-

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[5] Copies of the enlarged chart and nomograph may be obtained by writing to the Division of Pharmacology of the Food and Drug Administration.

tions, the method gains advantages which may be of considerable practical importance. Only about eight minutes are required for the calculations, approximately one-fourth of the time required for the rather involved calculations of Thompson's method. Moreover, the relative simplicity of the chart and nomograph enables quicker mastery of the method and decreases the probability of mistakes.

With regard to accuracy in estimating potencies, Thompson reported an average error of 2.9 percent for a group of assays entailing the injection of 10 to 14 doses and 1.7 percent for a group requiring the injection of 20 to 26 doses. The average actual error of the 16 dose method reported here was found to be 5.0 percent as compared with an average standard error of the assay of 5.1 percent (table 3).

The standard error of the assay shown in table 2 is an estimate of what the standard deviation of a series of potency determinations would be if the assays were run under identical conditions by one operator at one laboratory. This is to be distinguished from the standard error of the potency which is an estimate of how closely the assay can be duplicated by another operator at another laboratory. These two standard errors are identical *if* the method has been so standardized and collaborated upon that one assayist at one laboratory can check another assayist at another laboratory as closely as he can check himself.

It is believed that the method presented achieves the desirable objectives cited in the first paragraph of this paper and that it lacks the disadvantages which have been attributed to the present official method.

SUMMARY AND CONCLUSIONS

1. An objective method based on the use of a balanced experimental design and the use of a simple chart and nomograph for making the necessary calculations is presented for the biological assay of epinephrine.
2. The reliability of each assay may be easily estimated from the data obtained in that assay.
3. The method is simple and time-sparing and yet possesses a level of accuracy which should make it generally useful
4. A comparison of the true potencies with the estimated potencies showed the average error of nine assays of solutions of known potency to be 5.0 percent.

The technical assistance of Mr. Pete James and Mr. William D. Harkness is gratefully acknowledged.

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THE EFFECT OF AZOCHLORAMIDE ON SULFONAMIDE ACTIVITY[1]

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Two factors regarded as obstacles to the successful use of the sulfonamides have been (a) their inactivation by "antagonists" purported to be present in pus or tissue debris (1) and (b) the fact that some organisms become resistant to the action of these drugs. It was, therefore, a matter of considerable interest when Schmelkes and Wyss (2) reported that azochloramide, a compound which enhances the activities of the sulfonamides (3), was capable of inactivating sulfonamide antagonists and of making a resistant strain of *Escherichia coli* sensitive to sulfanilamide.

The initial object of the present investigation was to determine whether strains of resistant pneumococci could also be made sensitive to the sulfonamides by the addition of azochloramide to the test medium. When it was found that this was not the case, a more extended study was made of the so-called synergistic action of azochloramide and the sulfonamides. The details of this work are described below.

EXPERIMENTAL METHODS. Two strains of pneumococci and one strain of *Escherichia coli* were used as test organisms in this study. These were the parent type II CH strain of pneumococcus, its sulfonamide resistant derivative, type II CH (S), and the CH strain of *Escherichia coli*. The pneumococci were maintained by passage through mice three times each week, heart blood cultures being made in meat infusion blood broth. The strain of *Escherichia coli* was maintained by weekly subculture in meat infusion broth, the cultures being stored in the refrigerator between transfers. On the day preceding an experiment, the test organisms were transferred twice at 12 hour intervals in the basal medium used in the experiment. The second transfer, used as the source of the inoculum, was diluted in basal medium so that the desired number of organisms was contained in a volume of 1 ml.; in most tests a 10^{-4} dilution was employed.

The basal medium used for the pneumococci was beef heart infusion broth (4) to which two per cent freshly drawn, defibrinated rabbit blood was added on the day prior to the experiment; for the *Escherichia coli*, the media used were plain beef heart broth without enrichment and Sahyun's medium (5) to which 0.1 per cent casein hydrolysate (SMACO—vitamin free) was added. The drug containing broth was prepared as follows. Concentrated solutions of the sulfonamides[2] dissolved in the basal media were diluted serially and tubed in 8 ml. quantities; when the azochloramide solution and the inoculum were added to these tubes the final sulfonamide concentrations were 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.08 and 0.04 mgm. per cent. Sterilization was effected by autoclaving for 15 minutes at 15 pounds pressure.

The azochloramide solutions were prepared as follows. A 20 mgm. per cent solution was prepared in sterile distilled water and allowed to stand overnight in the refrigerator. This solution was always found to be sterile. It was diluted with sterile distilled water and 1 ml. quantities of the proper dilutions were added to the tubes of test broth immediately before inoculation.

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[1] This study was completed in 1942 but the pressure of war research delayed presentation of the data until the present time. Reporting of the data now seems justified by interest both in sulfonamide resistant organisms and in the treatment of infected wounds.

[2] At the time this study was initiated, the sulfonamides in most common use were sulfanilamide and sulfapyridine.

In the experiments with p-aminobenzoic acid, this substance was dissolved in the basal medium; the pH was adjusted to 7.6 and the solution was sterilized by autoclaving at 15 pounds pressure for 15 minutes. This stock solution was diluted serially with basal medium so that 1 ml. quantities could be added to each tube to form the desired concentrations. Special tubes of test broth in which the sulfonamide was dissolved and tubed in 7 ml. quantities were prepared for these experiments so that the final volume would again be 10 ml. after all constituents had been added.

The cultures were incubated at 37.5°C. Growth estimates made at intervals between 1 and 72 hours after inoculation were based on visual observations of turbidity in the experiments with *Escherichia coli* and turbidity and change in color of the red cell sediment in the experiments with the pneumococci. It should be noted that with these criteria the population of the cultures must reach five to ten million per ml. before there is visible evidence of growth.

Growth curve experiments were carried out in 50 ml. quantities of medium placed in milk dilution bottles. At various intervals these cultures were thoroughly mixed and 0.5 ml. samples were removed, diluted serially in meat extract broth and plated to determine the numbers of viable organisms present.

RESULTS. *A. Experiments with pneumococci. Sensitivity of pneumococci to azochloramide.* The effects of various concentrations of azochloramide on the parent and resistant strains of pneumococci are summarized in table 1. The data presented show that the two strains were equally susceptible to the action of this drug. Concentrations of 0.2 mgm. per cent or less had no apparent effect on growth; concentrations of 0.4 to 1.0 mgm. per cent delayed visible growth until 12 to 24 hours; 2.0 mgm. per cent prevented the appearance of growth throughout the 48 hour observation period.

Effects of azochloramide on the response of sensitive and resistant strains of pneumococci to sulfapyridine. The concentrations of azochloramide used in this study were selected on the basis of the data recorded in table 1 and represented levels of this substance which by themselves produced either none, slight or striking inhibition of growth. The effects of these various concentrations of azochloramide on the response of the parent and sulfonamide resistant strains of pneumococci to sulfapyridine are shown in table 2. Summarized briefly, the data show that concentrations of azochloramide which in themselves had no appreciable effect on the growth of the pneumococci likewise had no effect on the response of these organisms to sulfapyridine. Concentrations of azochloramide which in themselves produced a striking inhibition of growth caused a slight increase in the susceptibility of the organisms to the sulfonamide.

It is clearly evident from the data obtained in this experiment that even distinctly growth inhibitory concentrations of azochloramide were unable to abolish the resistance of the type II CH (S) strain. As a matter of fact, the effects of azochloramide on the susceptibility of the parent organism to sulfapyridine were somewhat greater than were those on the resistant strain.

Explanation of effects of azochloramide on sulfonamide activity. Although the results of the preceding experiment failed to provide any support for the idea that sulfonamide resistance is antagonized by azochloramide, they did show that this drug, in certain concentrations, effected a slight increase in sulfonamide activity. Since such concentrations of azochloramide were in themselves definitely growth inhibitory, the question was raised as to whether this apparent

TABLE 1

The effect of azochloramide on growth of sulfonamide susceptible and resistant strains of type II pneumococcus

ORGANISM	MGM. PER CENT AZOCHLORAMIDE	VISIBLE GROWTH			
		Hours of Incubation			
		8	12	24	48
Type II CH (Parent susceptible strain) Inoculum 1340/ml.	0	+	+	+	+
	0.05	+	+	+	+
	0.1	+	+	+	+
	0.2	+	+	+	+
	0.4	−	+	+	+
	0.6	−	−	+	+
	0.8	−	−	+	+
	1.0	−	−	+	+
	2.0	−	−	−	−
Type II CH (S) (Resistant strain) Inoculum 1300/ml.	0	+	+	+	+
	0.05	+	+	+	+
	0.1	+	+	+	+
	0.2	+	+	+	+
	0.4	−	+	+	+
	0.6	−	−	+	+
	0.8	−	−	+	+
	1.0	−	−	+	+
	2.0	−	−	−	−

TABLE 2

The combined effects of azochloramide and sulfapyridine on growth of susceptible and resistant strains of type II pneumococcus

ORGANISM	MGM. PER CENT AZOCHLORAMIDE	MGM PER CENT SULFAPYRIDINE REQUIRED TO INHIBIT GROWTH			
		Hours of Incubation			
		8	12	24	78
Type II CH (Parent susceptible strain) Inoculum 1670/ml.	0	2.5	2.5	5	5
	0.2	1.25	2.5	2.5	2.5
	0.4	—*	1.25	2.5	2.5
	1.0	—	—	1.25	1.25
Type II CH (S) (Resistant strain) Inoculum 1340/ml.	0	160	160	160	160
	0.2	160	160	160	160
	0.4	—	160	160	160
	1.0	—	—	80	80

* (—) indicates no growth even in the control tube which contained no sulfapyridine.

increase in activity was due merely to a reduction in the initial population of the culture. To test this hypothesis a study was made of the effects of azochloramide upon the numbers of viable organisms present in the cultures. The results of this study (fig. 1) showed that 0.2 mgm. per cent azochloramide, a concentration which did not affect the response of the pneumococci to sulfapyridine, had little effect on the populations of the cultures. On the other hand, 1.0 mgm. per cent azochloramide, a concentration which effected a slight increase in the sensitivity of the pneumococci to sulfapyridine, effected a sharp initial reduction in the culture population. This effect was only temporary, however, and rapid multiplication occurred within 4 to 8 hours.

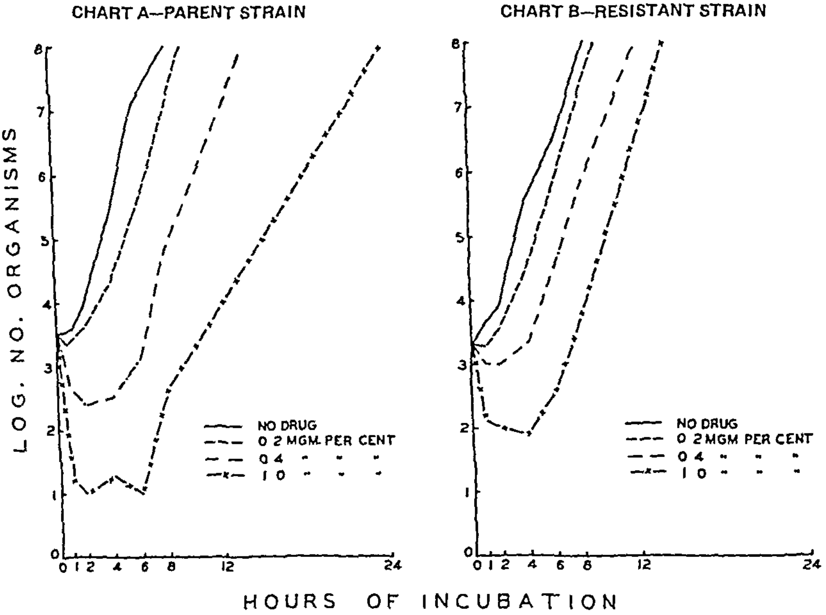

FIG. 1. THE EFFECTS OF AZOCHLORAMIDE ON THE GROWTH OF PARENT AND RESISTANT STRAINS OF TYPE II PNEUMOCOCCUS

Having demonstrated that azochloramide reduced the initial population of the culture, an experiment was carried out to determine whether such a reduction could explain the effects of this compound on sulfonamide sensitivity. The results of this experiment (table 3) show that a reduction in the initial population of the culture, equivalent to that which occurred in the presence of azochloramide, delayed the appearance of visible growth in essentially the same degree as when azochloramide was present in the medium, and effected a slight but definite increase in the sensitivity of the pneumococci to sulfapyridine.

B. Experiments with Escherichia coli. Since Schmelkes and Wyss (2) based their conception of the action of azochloramide on studies with *Escherichia coli*, it seemed important to examine the reactions of this latter organism as well as those of the pneumococci. Accordingly, experiments were carried out to determine (a) the effects of azochloramide on the sulfonamide sensitivity of *Escherichia coli* and (b) whether azochloramide could block the antagonism of sulfonamide activity produced by peptone or p-aminobenzoic acid.

Effects of azochloramide on sulfonamide sensitivity of Escherichia coli. The data in table 4 show the effects of various concentrations of azochloramide on the growth of *Escherichia coli* in a semi-synthetic medium low in sulfonamide inhibiting substances and in a complex medium rich in peptone. In both media azochloramide inhibited growth, but in order to produce the same effects more drug was required in the complex medium than in the semi-synthetic one. Thus in the semi-synthetic medium, 0.01 mgm. per cent azochloramide was without effect on growth; 0.05 to 0.2 mgm. per cent delayed the appearance of growth until 12 to 24 hours and 0.4 mgm. per cent inhibited visible growth throughout the 48 hour observation period. In the complex beef heart medium, however, the concentrations of azochloramide required for comparable effects were 0.05, 0.6 and 1.0 mgm. per cent respectively.

TABLE 3

The effect of size of inoculum on the activity of sulfapyridine against susceptible and resistant strains of type II pneumococcus

ORGANISM	INOCULUM ORGANISMS PER ML.	MGM. PER CENT SULFAPYRIDINE REQUIRED TO INHIBIT GROWTH			
		Hours of Incubation			
		8	12	24	48
Type II CH	20,000	2.5	5	5	5
(Parent susceptible	2,000	1.25	1.25	2.5	2.5
strain)	200	<0.6	1.25	1.25	1.25
	20	—*	<0.6	<0.6	1.25
	2	—	—	<0.6	<0.6
Type II CH (S)	32,000	160	160	160	160
(Resistant strain)	3,200	40	80	80	160
	320	—	40	80	80
	32	—	20	80	80
	3	—	—	80	80

* (—) indicates no growth even in the control tube which contained no sulfapyridine.

The effects of azochloramide on the activities of sulfanilamide and sulfapyridine were compared in the semi-synthetic medium and in the complex medium with the results shown in table 5. Summarized briefly, the data show that concentrations of azochloramide which by themselves had little or no growth inhibitory powers had little or no effect upon the response of *Escherichia coli* to the above sulfonamides. Concentrations of azochloramide which by themselves were definitely growth inhibitory produced apparent increases in sulfonamide sensitivity during the early hours of growth. At 48 hours, however, most of these apparent increases in sensitivity had disappeared.

Explanation of effects of azochloramide on sulfonamide activity. Following the lead obtained from the work with the pneumococci, experiments were carried out with *Escherichia coli* to determine whether the slight increases in sulfonamide sensitivity produced by azochloramide were due merely to a reduction in the population of the culture. Growth curve studies (fig. 2) show that concentrations of azochloramide which effected apparent increases in the sulfonamide sensitivity of *Escherichia coli* likewise effected initial reductions in the population of the culture. This occurred in both media, although in keeping with the observations of the preceding experiments, the effective amounts of azochloramide were greater in the complex than in the semi-synthetic medium.

TABLE 4

The effect of azochloramide on growth of Escherichia coli, Strain CH

MGM. PER CENT AZOCHLORAMIDE	VISIBLE GROWTH					
	Hours of Incubation					
	4	6	8	12	24	48
Tests in beef heart broth—inoculum 7800/ml.						
0	+	+	+	+	+	+
0.01	+	+	+	+	+	+
0.05	+	+	+	+	+	+
0.1	−	+	+	+	+	+
0.2	−	+	+	+	+	+
0.4	−	−	+	+	+	+
0.6	−	−	−	+	+	+
1.0	−	−	−	−	−	−
Tests in semi-synthetic medium—inoculum 2300/ml.						
0	+	+	+	+	+	+
0.01	+	+	+	+	+	+
0.05	−	−	−	+	+	+
0.1	−	−	−	−	+	+
0.2	−	−	−	−	+	+
0.4	−	−	−	−	−	−

As shown by the data in table 6, a mere reduction in inoculum such as was effected by the concentrations of azochloramide employed above, caused an apparent increase in sulfonamide sensitivity. This increase was fairly marked during the first 12 hours of incubation but was slight at the end of 48 hours. The effects were somewhat more striking with sulfapyridine than with sulfanilamide.

Effect of azochloramide on antagonism of sulfonamide activity by p-aminobenzoic acid. Studies with *Escherichia coli* in complex media (table 5) have already indicated clearly that azochloramide does not block the sulfonamide inhibiting properties of peptone. The effects of azochloramide on antagonism of sulfonamide activity by p-aminobenzoic acid have been summarized in table 7. The data in this table show that concentrations of azochloramide, which by themselves were only slightly growth inhibitory, had no effect on the p-aminobenzoic acid antagonism of sulfonamide activity. Concentrations which were distinctly growth inhibitory appeared to block the action of p-aminobenzoic acid during the first 24 hours of incubation. After this time, however, there was little or no effect on the activity of p-aminobenzoic acid.

TABLE 5

The combined effect of azochloramide and sulfonamide on growth of Escherichia coli, Strain CH

SULFONAMIDE	MGM. PER CENT AZOCHLORAMIDE	MGM. PER CENT SULFONAMIDE REQUIRED TO INHIBIT GROWTH				
		Hours of Incubation				
		6	8	12	24	48
Tests in beef heart broth—inoculum 7200 organisms/ml.						
Sulfanilamide.	0	20	160	>160	>160	>160
	0.05	10	40	>160	>160	>160
	0.1	10	40	160	>160	>160
	0.2	10	20	80	>160	>160
	0.4	—*	20	40	>160	>160
	0.6	—	—	40	>160	>160
Sulfapyridine.	0	2.5	20	20	20	160
	0.05	2.5	5	20	20	160
	0.1	1.25	5	20	20	160
	0.2	0.6	1.25	10	20	160
	0.4	—	0.6	2.5	20	80
	0.6	—	—	0.6	2.5	40
Tests in semi-synthetic medium—inoculum 3000 organisms/ml.						
Sulfanilamide	0		2.5	5	10	20
	0.012		2.5	5	10	20
	0.025		2.5	5	10	20
	0.05		—	2.5	5	20
	0.1		—	—	5	10
Sulfapyridine	0		0.3	0.3	0.6	2.5
	0.012		0.3	0.3	0.6	2.5
	0.025		0.15	0.15	0.6	2.5
	0.05		—	0.15	0.6	2.5
	0.1		—	—	0.6	1.25

* (—) indicates no growth even in the control tube which contained no sulfonamide.

COMMENT. The data presented above show that concentrations of azochloramide which of themselves have no growth inhibitory or only slight inhibitory action, have no effect on the response of either sensitive or resistant strains of pneumococci to the sulfonamides. Concentrations of azochloramide which of themselves are distinctly growth inhibitory effect apparently substantial increases in sulfonamide sensitivity during early hours of incubation but have only slight effects on sensitivity when observations are prolonged for 48 hours. These effects are of the same order for both the parent and the resistant strains and can be duplicated merely by reducing the inoculum of the culture to the same extent as that accomplished by the azochloramide. There is no indication that this substance abolishes the sulfonamide resistance of pneumococci. This is in accord with the observations of Stokinger, Charles, and Carpenter (6) on sulfonamide resistant gonococci.

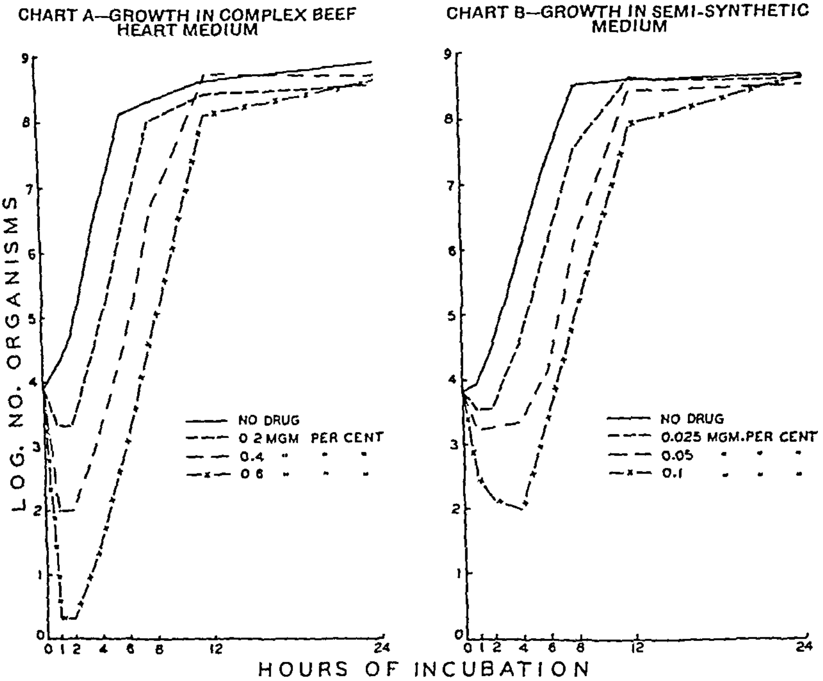

FIG. 2. THE EFFECTS OF AZOCHLORAMIDE ON THE GROWTH OF ESCHERICHIA COLI IN COMPLEX BEEF HEART BROTH AND IN SEMI-SYNTHETIC MEDIUM

Experiments with *Escherichia coli* likewise show that such increases in sulfonamide sensitivity as are produced by azochloramide may be ascribed to the reduction in population of the culture effected by the above substance. Azochloramide has no effect on inactivation of the sulfonamides by such substances as peptone and p-aminobenzoic acid.

The above findings offer no support for the contention of Schmelkes and Wyss (2) that azochloramide destroys sulfonamide resistance or abolishes antagonism of sulfonamide activity by peptone or p-aminobenzoic acid. Schmelkes and Wyss drew their conclusions from observations made after 15 or 16 hours incubation. It should be pointed out that the results of the current experiments indicate that observations at 15 hours would not disclose the early growth inhibiting action of azochloramide nor would they show such later outgrowth of the cultures in media containing both sulfonamides and azochloramide as has been observed in the present study. Thus, it seems probable that Schmelkes and Wyss have been led to erroneous conclusions because of insufficient data.

The findings of the present study make it appear doubtful that combined azochloramide-sulfonamide therapy possesses any particular advantage in treatment of infections with sulfonamide resistant organisms. Nor is there any indication that combined therapy has a peculiar value in lesions where large quantities of sulfonamide antagonizing substances may be present. Such beneficial effects as combined azochloramide-sulfonamide treatment seems to possess (7–11) may most likely be ascribed to apparent enhancement of sulfonamide activity by reduction in the numbers of organisms present in the lesion. Actually this reduction may be of considerable practical importance in the treatment of local infections.

TABLE 6

The effect of inoculum upon activity of sulfanilamide and sulfapyridine against Escherichia coli, Strain CH

SULFONAMIDE	INOCULUM ORGANISMS PER ML	MGM PER CENT SULFONAMIDE REQUIRED TO INHIBIT GROWTH				
		Hours of Incubation				
		6	8	12	24	48
		Tests in beef heart broth				
Sulfanilamide	9400	10	40	160	>160	>160
	940	—*	20	40	>160	>160
	94	—	10	40	>160	>160
	9	—	5	20	80	>160
	1	—	—	20	80	>160
Sulfapyridine	9400	0.6	5	20	160	160
	940	—	1.25	5	20	80
	94	—	0.6	2.5	10	40
	9	—	<0.3	1.25	20	40
	1	—	—	1.25	5	40
		Tests in semi-synthetic medium				
Sulfanilamide	3500	2.5	5	5	20	40
	350	—	2.5	5	10	20
	35	—	1.25	2.5	10	20
	3	—	—	2.5	10	20
	<1	—	—	1.25	10	20
Sulfapyridine	3500	0.08	0.3	0.3	1.25	2.5
	350	—	0.15	0.3	0.6	1.25
	35	—	0.08	0.3	0.6	1.25
	3	—	—	0.15	0.6	1.25
	<1	—	—	0.08	0.6	1.25

* (—) indicates no growth even in the control tube which contained no sulfonamide

TABLE 7

The effect of azochloramide upon the antagonistic action of p-aminobenzoic acid for sulfanilamide and sulfapyridine

Test organism: *Escherichia coli*, Strain CH, in Sahyun's medium—inoculum: 3500/ml.

MGM. PER CENT P-AMINOBENZOIC ACID	MGM. PER CENT AZOCHLORAMIDE	MGM. PER CENT SULFONAMIDE REQUIRED TO INHIBIT GROWTH				
		Hours of Incubation				
		6	12	24	48	72
Tests with sulfanilamide						
0	0	2.5	5	10	20	20
	0.012	2.5	5	10	20	20
	0.025	2.5	5	10	20	20
	0.05	—*	2.5	5	20	20
	0.1	—	—	5	10	10
0.012	0	40	40	80	160	>160
	0.012	20	40	80	160	>160
	0.025	2.5	20	40	160	>160
	0.05	—	10	40	80	160
	0.1	—	—	20	20	80
0.05	0	80	160	>160	>160	>160
	0.012	40	160	160	>160	>160
	0.025	2.5	20	160	>160	>160
	0.05	—	5	80	160	>160
	0.1	—	—	20	20	160
0.2	0	>160	>160	>160	>160	>160
	0.012	20	>160	>160	>160	>160
	0.025	2.5	160	>160	>160	>160
	0.05	—	2.5	>160	>160	>160
	0.1	—	—	160	160	160
Tests with sulfapyridine						
0	0	0.3	0.3	0.6	2.5	2.5
	0.012	0.3	0.3	0.6	2.5	2.5
	0.025	0.15	0.15	0.6	2.5	2.5
	0.05	—	0.15	0.6	2.5	2.5
	0.1	—	—	0.6	2.5	2.5
0.05	0	10	20	20	40	40
	0.012	10	10	20	40	40
	0.025	5	10	20	40	40
	0.05	—	10	20	40	40
	0.1	—	—	10	20	40
0.2	0	40	40	80	80	>160
	0.012	40	40	80	80	>160
	0.025	10	40	80	80	>160
	0.05	—	10	40	80	>160
	0.1	—	—	20	40	40
0.8	0	>160	>160	>160	>160	>160
	0.012	80	>160	>160	>160	>160
	0.025	40	>160	>160	>160	>160
	0.05	—	10	>160	>160	>160
	0.1	—	—	20	40	40

* (—) indicates no growth even in the control tube which contained no sulfonamide.

SUMMARY

Experiments have been carried out to determine the effects of azochloramide on the response of sensitive and resistant pneumococci to the sulfonamides. The results show that azochloramide concentrations which in themselves are distinctly growth inhibitory do effect slight increases in sulfonamide sensitivity. These increases are of the same order for both sensitive and resistant strains and can be accounted for by the reduction in the initial populations of the cultures produced by azochloramide. This substance does not abolish the sulfonamide resistance of pneumococci.

Experiments carried out with *Escherichia coli* likewise show that concentrations of azochloramide which are distinctly growth inhibitory effect a slight increase in sulfonamide sensitivity. Again the increase in sensitivity appears to be due to a reduction in the initial population of the culture.

Additional experiments with *Escherichia coli* show that antagonism of sulfonamide activity by peptone or p-aminobenzoic acid is not blocked by azochloramide.

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VITAMIN A CONCENTRATES, MARINE OIL FRACTIONS, AND VITAMIN K IN THE TREATMENT OF EXPERIMENTAL RENAL HYPERTENSION[1]

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Since vitamins are known to play important rôles in cellular metabolism which must be altered in the kidney of experimental renal hypertensive animals and since there are clinical reports of a certain effectiveness of vitamin A concentrates in essential hypertension in man (1), we undertook a study of the possible effect of various vitamin preparations, including vitamin A concentrates, on the blood pressure of renal hypertensive dogs. We previously reported that vitamin C, a vitamin B concentrate, and vitamin E were without effect on this form of experimental hypertension (2). More recently we have found dried brewer's yeast[2] in a daily oral dose of 2 gm. per kilo. for six months to be without therapeutic effect in two hypertensive dogs (3). In a preliminary communication, we also reported that one lot of a vitamin A concentrate in sesame oil showed an excellent antihypertensive effect in a daily oral dose of 1 cc. for three months followed by 2 cc. for three months (200,000 I.U. of vitamin A per cc.) but that the therapeutic activity was not due to vitamin A since two other lots of presumably the same concentrate, as well as a vitamin A alcohol in equivalent dose, were inactive antihypertensively (4). In view of these findings and the reports by Grollman and his co-workers confirming the irregular presence of an orally effective antihypertensive principle in fish liver and other marine oils (5), we have studied additional vitamin A concentrates and also certain fish liver oil and marine oil fractions in renal hypertensive dogs in a further effort to demonstrate and identify this antihypertensive principle. We likewise studied the possible antihypertensive effect of vitamin K in dogs. While in progress this study was stimulated by reports of the effectiveness of vitamin K (and other quinones) in experimental renal hypertension (6, 7). Our findings with the additional vitamin A concentrates, the fish liver and marine oil fractions, and vitamin K in the treatment of experimental renal hypertension are summarized in this report.

METHODS. Dogs weighing 10–15 kilo. were rendered hypertensive by the classic Goldblatt technique and showed a good to excellent grade of hypertension for a minimum period of five months prior to use as assay animals. Mean blood pressures were determined two to three times weekly by direct femoral arterial puncture. Body weights, blood urea nitrogens and urines were checked at monthly intervals. These observations were continued for a minimum of six months following treatment.

RESULTS. I. *Vitamin A Concentrates.* Therapeutic assay of a fourth lot of presumably the same vitamin A concentrate[3] previously reported as excellently

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[2] Supplied by Mead Johnson and Company.

[3] Supplied by the Winthrop Chemical Company as Afaxin.

antihypertensive for one lot and inactive antihypertensively for two other lots (4), demonstrated no effect in one dog and a slight but significant decrease in blood pressure in two other animals. Each animal received 2 cc. of the concentrate by mouth daily for six months (200,000 I.U. of vitamin A per cc.).

Another vitamin A concentrate[4] administered orally for four months and intramuscularly for an additional four months produced a slight but significant decrease in blood pressure in one hypertensive dog. The daily oral dose of the concentrate in sesame oil was 2 cc. (100,000 I.U. per cc.) and the daily intramuscular dose in "injectable oil" was 2 cc. (100,000 I.U. per cc.).

A third vitamin A concentrate[5] in sesame oil was inactive antihypertensively in one dog in a daily oral dose of 2 cc. (100,000 I.U. per cc.) for four months.

These results as well as those previously reported for the first vitamin A concentrate are summarized in table I.

II. *Fish Liver and Marine Oil Fractions.* Therapeutic assay of the residue oil from vitamin A stills[6] in a daily oral dose of 2 cc. for four months, showed no effect in one hypertensive dog. A distillation residue from mixed fish body oils[7] from which most of the fatty acids had been removed (designated as "blended pitch") given to hypertensive dogs in a daily oral dose of 4–6 gm. for four months proved inactive antihypertensively. A distillation residue from marine fish liver oils (designated as "marine pitch") was administered to four hypertensive dogs in a daily oral dose of 4–12 gm. for four months without significant effect on blood pressure. The first residue contained approximately 1000 I.U. of vitamin A per cc.; the other two were devoid of vitamin A. The results are summarized in table 1.

III. *Vitamin K.* The possible antihypertensive activity of this vitamin[8] was assayed in six dogs. One animal received vitamin K in sesame oil in a dose of 30 mg. daily by mouth for three months. Four dogs were given vitamin K powder mixed with food in a daily dose of 60 mg. for six months. The sixth dog received the latter dose intramuscularly in sesame oil for six months. None of these animals showed any significant change in their hypertensions. The results are summarized in table 1.

DISCUSSION. Of the four lots of the first vitamin A concentrate studied excellent antihypertensive activity and slight antihypertensive activity were found in only the first and fourth lots respectively. No antihypertensive effect was found in the second and third lots. Slight antihypertensive activity and no antihypertensive activity were demonstrated in the second and third concentrates respectively. Obviously this activity is inconstantly present in vitamin A concentrates and in variable amounts wholly unrelated to vitamin A potency.

Our failure to demonstrate the activity in the residue from vitamin A stills and in the residues resulting from the distillation of mixed fish body oils and marine fish liver oils suggests either that the active principle is volatile or is destroyed during distillation. The recent report of Grollman (5) indicates that the principle distills over. Obviously much work remains to be done in order to determine the

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[4] Supplied by the E. A. Brewer Company as Avita.

[5] Supplied by the Nion Corporation.

[6] Supplied by the Distillation Products, Inc.

[7] Supplied by the Werner G. Smith Company.

[8] Supplied by the Winthrop Chemical Company as Kappaxin.

TABLE 1

Vitamin A concentrates, marine oil fractions and vitamin K in the treatment of experimental renal hypertension

			NO. OF DOGS	DOSE PER DAY*	LENGTH OF TREATMENT	BLOOD PRESSURE RANGE (MM. HG.)		ANTI-HYPERTENSIVE EFFECT†
						Before treatment	At end of treatment	
Vitamin A Concentrates	#1	Lot 1	3	1–2 cc.	6 mo.	140–180	100–115	+++
						190–205	130–150	+++
						150–170	125–145	++
		2	3	2 cc.	2–6 mo.	150–170	140–160	−
						170–190	170–185	−
						150–180	150–180	−
		3	1	2 cc.	4 mo.	155–180	140–150‡	−
		4	3	2 cc.	6 mo.	150–170	130–150	+
						170–185	175–190	−
						155–185	140–165	+
	#2		1	2 cc. orally and I.M.	8 mo.	150–160	135–140	+
	#3		1	2 cc.	4 mo.	170–180	165–180	−
Fish Liver and Marine Oil Fractions	Residue oil		1	2 cc.	4 mo.	155–175	155–180	−
	Blended pitch		2	4–6 gms.	4 mo.	160–170	160–175	−
						200–230	220–240	−
	Marine pitch		4	4–12 gms.	4 mo.	170–190	150–170‡	−
						175–190	175–185	−
						145–170	155–180	−
						175–190	175–190	−
Vitamin K	In Sesame oil		1	30–60 mgms.	3 mo.	140–160	130–160	−
	Crystalline		4	60 mgm.	6 mo.	150–170	145–165	−
						160–180	160–180	−
						160–185	160–185	−
						140–160	130–155	−
	In Sesame oil		1	60 mgm.	6 mo.	160–180	160–170	−

* Oral unless otherwise stated.

† Antihypertensive effect.

‡ Spontaneous decrease unrelated to treatment.

− No antihypertensive effect.

+ Slight antihypertensive effect.

++ Moderate antihypertensive effect.

+++ Excellent antihypertensive effect.

best sources of this orally effective antihypertensive principle and the optimal conditions for its separation, concentration, and identification.

Our findings with vitamin K are at variance with those reported by Oppenheimer et al. (6) and Schwarz and Ziegler (7) for hypertensive rats. Certainly our results do not warrant trial of this vitamin in the treatment of human hypertension as suggested by the latter.

SUMMARY

1. Vitamin A concentrates inconstantly contain variable amounts of an orally effective antihypertensive principle demonstrable by assay on renal hypertensive dogs. This principle was not present in the assayed residues from marine oils subjected to distillation in the preparation of vitamin A concentrates and other products. The antihypertensive principle definitely is not vitamin A. More work is necessary in order to separate, purify, and identify this principle.

2. Vitamin K is without antihypertensive activity in renal hypertensive dogs.

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THE INACTIVATION OF PITOCIN AND PITRESSIN BY HUMAN PREGNANCY BLOOD

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It has been shown that approximately 65% of patients with eclampsia and preeclampsia are hypersensitive to the pressor action of Pituitrin, Pitressin and Pitocin (1, 2, 3). This hypersensitivity could be due either to an increased sensitivity of the effector cells or to a diminished destruction or inactivation of the pressor substance. The primary aim of this investigation was to test this latter hypothesis. A secondary aim was to determine whether this hypersensitivity to the pressor action also extended to the oxytocic activity.

In numerous experiments on the nature of the posterior pituitary substances it has been found that they can be inactivated by certain enzymes and tissue preparations. Heller and Urban (4) showed that the anti-diuretic hormone is inactivated by dog blood and serum, rabbit and human blood but not by the cerebro-spinal fluid. The time for inactivation varied from one and one-half to five hours at 39°C. when 50 milliunits of pituitrin was added to one cc. of the tissue.

Larson (5) determined that the pressor component as tested on the barbitalized cat is inactivated by incubation at 37°C. for 24 to 72 hours with extracts of liver, kidney, muscle or blood from dogs, cats or rabbits. An amino-peptidase and a dipeptidase obtained from dog tissue were also found to inactivate the vasopressin. In an effort to separate the oxytocic and vaso-pressor fractions of pituitary by enzymatic hydrolysis Christlieb (6) found that they were equally inactivated by 24-hour incubation at 37°C. by various concentrations of tissue extracts. She used intestine, liver, kidney and muscle and tested the responses on isolated sheep uteri and on the blood pressure of the rat.

Feteke (7) found that the blood serum from pregnant women was more active in inactivating oxytocin than was non-pregnant blood serum. He used the isolated guinea pig uterus as the test organ and showed that one unit of oxytocin was inactivated by two-hour incubation with pregnancy serum. This could also be demonstrated on the human uterus. Ten units of pituitrin incubated for 15 hours with 10 cc. of pregnancy serum produced no uterine contractions while a control 10-unit dose produced very violent contractions. Feteke also found that using very sensitive rat uteri normal serum produced contraction while pregnancy serum did not. This indicates the presence in normal serum of an oxytocic substance probably of posterior pituitary origin.

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Human pregnancy blood can also inactivate the vaso-pressor fraction. This has been shown by Werle and Kalvelage (8) using the rat blood pressure. The inactivating substance appears at about the second month of gestation and increases in amount up to term and then decreases, finally disappearing about three weeks after delivery. This inactivating substance was not found in the fetal blood.

METHODS. In a series of patients (table 1), some normal and others showing symptoms of toxemia, the uterine contractions were recorded by an intra-uterine balloon and optical manometer as previously described (9). Commercial Pitocin and Pitressin were used in doses ranging from 0.1 to 2 units. These were administered intravenously in order that a clear, rapid uterine response could be obtained. In the toxemic patients labor was induced by the uterine balloon and pituitary preparations, while in the normal patients the balloon was not inserted until labor was well established.

TABLE 1

Pregnant patients

PATIENT	AGE	GRAV.	PARA.	DURATION OF PREGNANCY	B.P.	SYMPTOMS ON ADMISSION		
						Edema	Albuminuria	Headache and/or visual disturb.
				months				
R. H.	18	I	0	Term	115/70	–	–	–
R. G.	16	I	0	Term	115/70	–	–	–
M. M.	31	VI	V	3	140/80	++	–	+
E. W.	33	V	V	5	155/90	++	–	+
M. S.	30	XIII	XIII	6	120/76	++	–	+
B. M.	35	XII	XI	7½	170/130	–	–	–
E. B.	20	I	0	8	190/85	+	+++	–
C. H.	21	VI	IV	8½	140/100	++	–	+
A. B.	25	VI	V	Term	130/100	++	+	+

After a sufficient control record (40 min. to 3 hrs.) was obtained a small dose of the substance to be tested was injected in 10 to 40 cc. of istonic saline solution. If the response was suitable this dose was used as the control. If not, a slightly larger dose was given. In the later months of gestation 0.1 unit of Pitocin was found to give a suitable response. In the early months, up to one unit was necessary. The dose of Pitressin (necessary to produce a uterine response) varied from 0.3 to 2.0 units. In the case of Pitressin while only the oxytocic activity was recorded other signs of activity such as nausea, vomiting, defecation and changes in intestinal activity, blood pressure and pulse rate changes were noted.

Blood was drawn from the patient, heparinized and the test dose of pituitary substance added. After a period of incubation at 37°C. this blood was reinjected. A control dose was then again administered, allowing at least 40 minutes between each injection.

In some of the patients the Pitressin and Pitocin were administered after incubation in blood from cross-matched donors. These donors included normal males, a non-pregnant female, and a 7-day post partum patient.

RESULTS AND DISCUSSION. Figure 1 illustrates the results obtained. The results of incubating Pitocin in pregnancy blood are given in Table 2, and in non-pregnancy blood in Table 3. Tables 4 and 5 give the results with Pitressin. all cases where the oxytocic action of Pitressin was nullified all other sympto of Pitressin activity were also removed.

The oxytocic components of Pitocin and Pitressin are rapidly inactivated by short period of incubation in blood obtained from pregnant humans. T

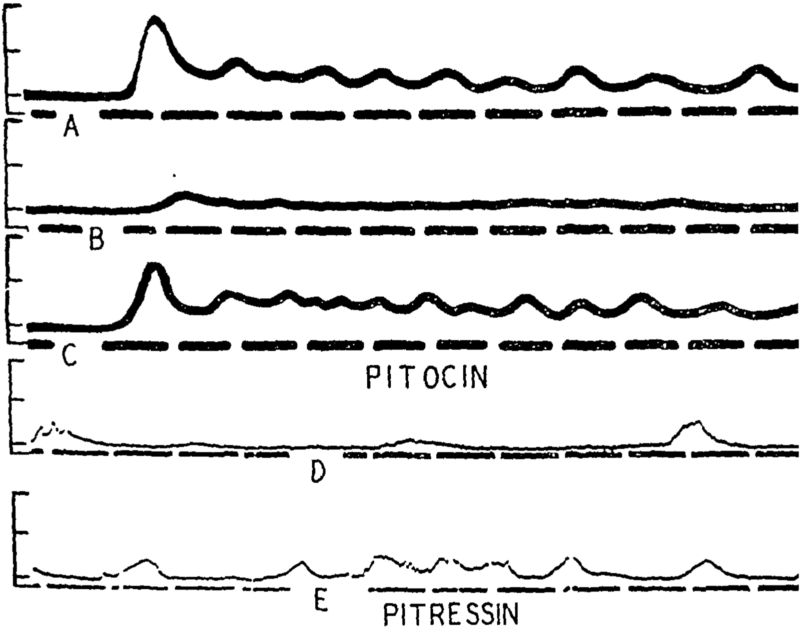

Fig. 1. Uterine Pressure Records Showing the Results of Incubating Pitocin
Pitressin in Human Pregnancy and Non-pregnancy Blood

Each division on the pressure standard is equal to 50 mm. Hg. Time, one minute.

Pitocin—Record from patient M. S. A: Pitocin, one unit, in 40 cc. saline injected int venously at signal. B: Pitocin, one unit, incubated in 40 cc. of the patient's blood fo min. and injected at signal. C: Pitocin, one unit, incubated for 15 min. in 50 cc. of m blood (R. W.) injected at signal. Note the almost complete inactivation of the oxyto effect of Pitocin when incubated in the pregnancy blood.

Pitressin—Record from patient B. M. D: Pitressin, 0.75 unit, incubated for 15 min 10 cc. of the patient's blood. Note the complete inactivation. E: Pitressin, 0.75 u incubated for 15 min. in 10 cc. of male blood (G. C.). This Pitressin response is equal to control response which is not illustrated.

pressor component is also inactivated since after incubation Pitressin does show any of its usual smooth muscle effects. Previous work has shown t while many tissues including blood can inactivate the posterior pituitary s stances a long period of incubation is required. We have found that hum pregnancy blood can very rapidly inactivate these substances. The obser maximal rate of inactivation was 0.025 unit of Pitocin per cc. of blood in 20 minutes and 0.1 unit of Pitressin per cc. of blood in 20 min. These values are probably not the absolute maximums since no attempt was made to determine these.

TABLE 2

Pitocin in pregnancy blood

PATIENT	GESTATION PERIOD	DIAGNOSIS	PITOCIN DOSE	BLOOD	INCUBATION TIME	RESULT
	months		*units*	*cc.*	*minutes*	
M. M.	3	Toxemia	0.5	50	30	Not inactivated
M. S	6	Toxemia	1.0	40	15	Inactivated
B M.	7½	Hypertension	0.1	10	20	Inactivated
C. H.	8½	Toxemia	0 1	10	20	Inactivated
R. G.	9	Normal labor	0.1	9	25	Inactivated
R H	9	Normal labor	0.1	10	30	Inactivated

TABLE 3

Pitocin in non-pregnancy blood

DONOR	TYPE	TEST PATIENT	PITOCIN DOSE	BLOOD	INCUBATION TIME	RESULT
			units	*cc*	*minutes*	
R W	Male	M S	1 0	50	15	Not inactivated
G C	Male	B. M.	0 1	10	20	Not inactivated
R. H	Male	B M	0 1	10	20	Not inactivated
A W	Female	E B	0 1	10	20	Not inactivated
M P	7 days post-partum	E B	0 1	10	20	Inactivated

TABLE 4

Pitressin in pregnancy blood

PATIENT	GESTATION PERIOD	DIAGNOSIS	PITRESSIN DOSE	BLOOD	INCUBATION TIME	RESULT
	months		*units*	*cc*	*minutes*	
M. M	3	Toxemia	1 0	50	20	Not inactivated
E. W	5	Toxemia	0 4	50	20	Inactivated
			0 4	20	25	Inactivated
B M	7½	Hypertension	0 75	10	15	Inactivated
C. H	8½	Toxemia	0 3	10	20	Inactivated
			2 0	20	20	Inactivated
A B	9	Toxemia	0 12	20	20	Inactivated
			1 2	20	20	Inactivated
R G.	9	Normal labor	0 25	15	60	Inactivated
R H.	9	Normal labor	0 5	20	13	Inactivated

While the exact nature of the inactivating substance is unknown it is probably an enzyme. Amino-peptidase and dipeptidase from various animal tissues (5) and from yeast (10) have been found to inactivate oxytocin and vaso-pressin. Extract of hypertensive kidney can inactivate Pitocin but pepsin and rennin free from hypertensinase cannot (10).

This ability to rapidly inactivate the posterior pituitary substances appears some time after the third month of pregnancy, before the fifth month of pregnancy and persists until at least 7 days post partum (see table 6). It is not present in males, non-pregnant females and early pregnant females (under 3 months). These observations suggest that some of the abortions from increased uterine activity may occur because the individual fails to increase her capacity to destroy posterior pituitary material before or by the time the uterus becomes more responsive to the oxytocic material. Robson (11) has shown that during the 12th to 24th week of pregnancy the human uterus becomes more sensitive to pituitary oxytocic substances. These observations also serve as additional evidence that the posterior pituitary is not the causative agent of parturition in the human.

TABLE 5

Pitressin in non-pregnancy blood

DONOR	TYPE	TEST PATIENT	PITRESSIN DOSE	BLOOD	INCUBATION TIME	RESULT
			units	*cc.*	*minutes*	
G. C.	Male	B. M.	0.75	10	15	Not inactivated
A. W.	Female	E. B.	0.3	10	20	Not inactivated
M. P.	7 days post-partum	E. B.	0.3	10	20	Inactivated

TABLE 6

Inactivation of Pitocin and Pitressin in pregnancy and non-pregnancy

DONOR	TYPE	GESTATION PERIOD	PITOCIN	PITRESSIN
		months		
R. W.	Male		Not inactivated	
G. C.	Male		Not inactivated	Not inactivated
R. H.	Male		Not inactivated	
A. W.	Female	0	Not inactivated	Not inactivated
M. M.	Female	3	Not inactivated	Not inactivated
E. W.	Female	5		Inactivated
M. S.	Female	6	Inactivated	
B. M.	Female	7½	Inactivated	Inactivated
C. H.	Female	8½	Inactivated	Inactivated
A. B.	Female	9		Inactivated
R. G.	Female	9	Inactivated	Inactivated
R. H.	Female	9	Inactivated	Inactivated
M. P.	Female	7 days post-partum	Inactivated	Inactivated

The data also show that the increase in uterine activity following 0.1 unit of Pitocin is much greater in two toxemic patients than in the normal controls (see fig. 2). The series is too small to justify definite conclusions, but it does indicate that toxemic patients are hypersensitive to the oxytocic activity of pituitary substances.

The blood of toxemic as well as normal pregnant humans can inactivate rapidly Pitocin and Pitressin. This indicates that the hypersensitivity of pre-eclamptic and eclamptic to vaso-pressin and oxytocin is not due to a diminished ability to inactivate these substances.

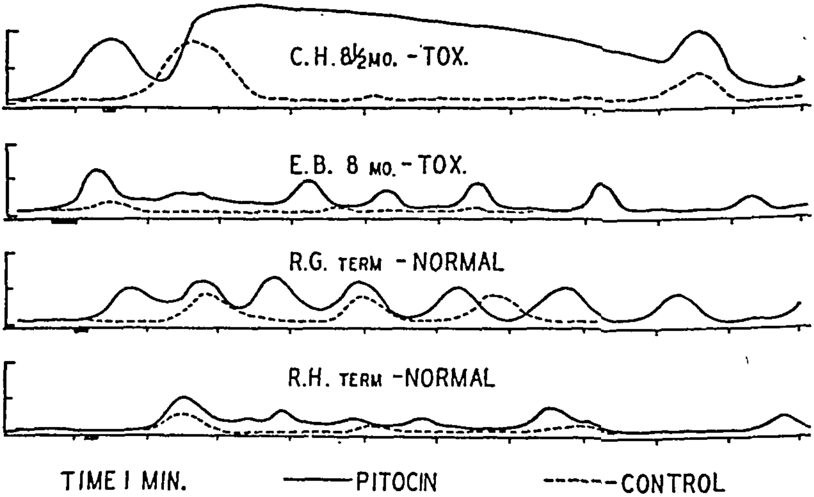

FIG. 2. UTERINE PRESSURE RECORDS SHOWING THE RESPONSE TO 0.1 UNIT OF PITOCIN IN FOUR PATIENTS

Each division of the pressure standard equal to 50 mm. of Hg. Time, one minute.

The broken line is a reconstruction of the control uterine activity just before the administration of the Pitocin. The solid line represents the uterine response to 0.1 unit of Pitocin injected at signal. The hypersensitivity to Pitocin is obvious in the first toxemic patient (C. H.). The response to Pitocin shown by patient E. B. does not at first appear excessive, but when the control uterine activity in this patient is compared to the controls in the normal patients the response to Pitocin seems greater than in the normals.

SUMMARY AND CONCLUSIONS

1. Blood from humans from about the fifth month of pregnancy to at least seven days post partum can rapidly inactivate Pitocin and Pitressin as tested by their oxytocic action on the pregnant human uterus. These observations serve as additional data that the posterior pituitary is not the causative agent of parturition in humans.

2. Blood from males, non-pregnant females and early pregnancy cannot inactivate these substances as rapidly.

3. Toxemic patients appear to be more sensitive to the oxytocic action of Pitocin.

4. Toxemic patients can inactivate Pitocin and Pitressin as rapidly as normal patients. The hypersensitivity of pre-eclamptics and eclamptics to vaso-pressin and oxytocin is not associated with a diminished ability of the blood to inactivate these substances.

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